

Cold-Shock Regulatory Elements, Constructs Thereof, and Methods of Use

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5 This application is a continuation-in-part of Serial No. 09/293,427, filed April 16, 1999, entitled "Method and Constructs for Inhibiting Protein Expression in Bacteria," which is a continuation-in-part of Serial No. 08/769,945, filed December 19, 1996, entitled "Method and Constructs for Inhibiting Protein Expression in Bacteria," which is a continuation -in-part of Serial No. 08/203,806, filed March 1, 1994, entitled "Nucleic Acids Sequence, Stress-Induced Proteins and Uses Thereof," which corresponds to parent application Serial No. 07/852,013, ^{filed 03/09/92} This application also claims the priority of provisional application Serial No. 60/096,938, filed August 20, 1998, entitled "The 5'Untranslated Region of the Cold-Shock *cspA* Gene Regulates Translation Efficiency in Addition to mRNA Stability," and U.S. ^{filed 07/12/99,} provisional application Serial No. 60/143,380, entitled "Translational Enhancement by an Element Downstream of the Initiation Codon in *Escherichia coli*."

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BACKGROUND OF THE INVENTION

15 **1. Field of the Invention**

The invention relates to the control of bacterial gene expression, especially the regulation of bacterial gene expression under conditions of physiological stress. More specifically, the invention relates to the regulation of bacterial gene expression under conditions of physiological stress that induce the cold shock response of a bacterium.

20 **2. Description of the Related Art**

The regulation of bacterial gene expression occurs at many levels, including transcriptional control, or control of the synthesis of mRNA from a given gene; translational control, or the regulation of the efficiency by which the mRNA is translated into polypeptide sequence by the ribosome; and mRNA stability, or the efficiency at which a given mRNA

population within the cell is degraded and rendered inactive. The control of bacterial gene expression under conditions of physiological stress that elicit the cold shock response of the bacterium involve regulation at all three of the levels described above.

The response of bacteria to physiological stress involve the tightly controlled expression of a small number of genes that function to allow the cell to adapt to and function under stress conditions. For example, when bacterial cells are exposed to temperatures above the normal physiological temperature for that organism, a set of genes, designated the heat shock genes, are expressed. This response to elevated temperatures is well known and described in the prior art. Conversely, when bacterial cells are exposed to lower than physiological temperatures, a different set of genes, designated as cold shock (cs) genes, are expressed. Expression of the cs genes allow the cell to first adapt to the physiological stress, and subsequently grow under conditions of physiological stress. This invention relates to the specific processes that regulate the expression of cs genes.

When a culture of *Escherichia coli* is shifted from 37°C to 15 or 10°C, a number of proteins, called cold-shock proteins, are transiently induced during its growth lag period (Jones *et al.*, 1987; for review, see Thieringer *et al.*, 1998; Yamanaka *et al.*, 1998). *cspA*, consisting of 70 amino acid residues, has been identified as a major cold-shock protein (Goldstein *et al.*, 1990) and its three-dimensional structure has been determined by both X-ray crystallography (Schindelin *et al.*, 1994) and nuclear magnetic resonance spectroscopy (Newkirk *et al.*, 1994; Feng *et al.*, 1998) to consist of a five-antiparallel β -stranded structure. *cspA* can bind to single-stranded DNA and RNA without high sequence specificity and has been proposed to function as an RNA chaperone at low temperature (Jiang *et al.*, 1997).

To date, more than 50 proteins homologous to CspA have been identified in a large varieties of prokaryotes. Moreover, a region called cold-shock domain of eukaryotic Y-box

protein family, such as human YB-1 and *Xenopus* FRGY-2, shares more than 40% identity with *E. coli* *cspA* (for review, see Wolffe *et al.*, 1992), indicating that the cold-shock domain is well conserved throughout evolution. In *E. coli*, nine genes encoding *cspA*-like proteins, *cspA* to *cspI*, have been identified (for review, see Yamanaka *et al.*, 1998). Among them, *cspA*, *cspB* and *cspG* are cold-shock inducible (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Nakashima *et al.*, 1996) and interestingly, *cspD* is induced during stationary phase and upon nutrition starvation (Yamanaka and Inouye, 1997). It was proposed that the large *cspA* family of *E. coli* may have a function to respond to different environmental stresses (for review, see Yamanaka *et al.*, 1998).

cspA expression is transiently induced upon cold shock during the growth lag period called acclimation period. This period is considered to be required for cells to adapt to a new environmental condition. Indeed, during the acclimation period proteins involved in translation such as CsdA (Jones *et al.*, 1996), RbfA (Jones and Inouye, 1996) and CspA (Goldstein *et al.*, 1990) are specifically produced, which are considered to play important roles in enhancing translation efficiency for non-cold-shock proteins at low temperature (for review, see Yamanaka *et al.*, 1998). Among these cold-shock proteins, *cspA* has been quite extensively investigated for the mechanism of its cold-shock induction (for review, see Yamanaka *et al.*, 1998). The *cspA* promoter is highly active at 37°C, even if CspA is hardly detected at this temperature (Fang *et al.*, 1997; Mitta *et al.*, 1997). Even if the *cspA* promoter was replaced with the *lpp* promoter, a constitutive promoter for a major outer membrane protein, *cspA* expression is still cold-shock inducible (Fang *et al.*, 1997), indicating that the *cspA* induction at low temperature occurs mainly at levels of mRNA stability and its translation. The *cspA* promoter, however, contains an AT-rich upstream element (UP element) (Ross *et al.*, 1993) immediately upstream of the -35 region (Fang *et al.*, 1997;

Goldenberg *et al.*, 1997; Mitta *et al.*, 1997), which is considered to play an important role in efficient transcription initiation at low temperature. It has been demonstrated that the *cspA* mRNA becomes extremely stable upon cold shock, indicating that the mRNA stability plays a crucial role in cold-shock induction of *cspA* (Brandi *et al.*, 1996; Goldenberg *et al.*, 1996; Fang *et al.*, 1997).

An important and unique feature of the *cspA* mRNA is its unusually long 5'-untranslated region (5'-UTR) consisting of 159 bases (Tanabe *et al.*, 1992). This feature is also shared with other Class I cold-shock genes, which are dramatically induced after temperature downshift (for review, see Thieringer *et al.*, 1998), such as *cspB* (Etchegaray *et al.*, 1996) and *cspG* (Nakashima *et al.*, 1996). The 5'-UTR is considered to play a crucial role in the cold-shock induction of *cspA* (Brandi *et al.*, 1996; Jiang *et al.*, 1996; Goldenberg *et al.*, 1996; Bae *et al.*, 1997; Fang *et al.*, 1997; Goldenberg *et al.*, 1997; Mitta *et al.*, 1997).

Furthermore, it was recently shown that the 14-base downstream box (DB) located 12 bases downstream of the translation initiation codon of the *cspA* mRNA, which is partially complementary to a region called anti-downstream box of 16S rRNA (Sprengart *et al.*, 1996), plays an important role in efficient translation at low temperature (Mitta *et al.*, 1997). This region of the RNA sequence designated as the downstream box (DB) is complementary to bases 1469-1483 within the *E. coli* 16S rRNA (anti-DB sequence). It is speculated that formation of a duplex between the DB and anti-DB of 16S rRNA is responsible for translational enhancement (Sprengart *et al.*, 1996). The DB sequence has also been implicated in the translation of the λ cI mRNA, a mRNA that lacks any untranslated region and the SD sequence (Shean and Gottesman, 1992; Powers *et al.*, 1988). Interestingly λ cI translation was enhanced at 42°C in a temperature sensitive strain in which the amount of ribosomal protein S2 decreased at 42°C. It was proposed that the anti-DB sequence in S2

deficient ribosomes indirectly becomes more accessible to DB, resulting in enhancement of translation initiation of the λ cI mRNA. However, the role of the DB in λ cI translation initiation was disputed by Resch and coworkers (Resch et al., 1996). These authors constructed *lacZ* translational fusions with the λ cI gene to test the DB function. Since a
5 deletion of 6 bases encompassing a portion of the DB sequence did not reduce the formation of the translation initiation complex, they disputed the existence of DB. Despite these elusive roles of DB (Sprengart and Porter, 1997) we have shown that the presence of a DB sequence in cold-shock mRNAs plays an important role in translation efficiency, and proposed that the DB is involved in the formation of a stable initiation complex at low temperature before the
10 induction of cold-ribosomal factors (Mitta et al., 1997). Thus, *cspA* expression is regulated in a complex manner at levels of transcription, mRNA stability and translation.

Here, we constructed a series of deletion mutations in the 5'-UTR of *cspA* and analyzed their effects on *cspA* expression by examining the amount of mRNA, mRNA stability and translational efficiency. It was discovered that besides mRNA stability, the 5'-
15 UTR plays a major role in translation efficiency of the *cspA* mRNA to enhance *cspA* expression upon cold shock. Specific regions of the 5'-UTR have been found to mediate the regulatory processes described above.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the plasmid pJJG78 and the effects of the *cspA* upstream region on the chromosomal *cspA* expression and the synthesis of other cellular proteins: (A) pJJG78. (B) the effects of the *cspA* upstream region on the chromosomal *cspA* expression and the synthesis of other cellular proteins.

Fig. 2 shows the prolonged expression of CspA and inhibition of cold-shock adaptation by pJJG78 and pUC19-600.

Fig. 3 shows deletion analysis of the *cspA* upstream region for the *cspA* derepression function and inhibition of cold shock adaptation.

Fig. 4 shows the level of transcripts from the chromosomal and plasmid *cspA*.

Fig. 5 shows the requirement for the transcription of the 5' untranslated region of the *cspA* mRNA for the prolonged expression of *cspA* and inhibition of cold-shock adaptation.

Fig. 6 shows the effects of over-production of the 5' untranslated region of the *cspA* mRNA on the production of other cold-shock proteins and non-cold-shock protein.

Fig. 7 shows the effects of co-overproduction of *cspA* together with the 5' untranslated region of the *cspA* mRNA on the cold-shock response.

Fig. 8 shows the effects of overproduction of the first 25-base sequence of the *cspA* 5' UTR on CspA production.

Fig. 9. shows cold-shock induction of β -galactosidase: (A) Construction of *cspA-lacZ* fusions. The wild-type *cspA* is shown on the top. The *cspA-lacZ* fusion in each expression plasmid is shown from the 5' end of the *cspA* promoter upstream region to *lacZ*. Nucleotide numbers are given starting from the transcription initiation site as +1, determined by Tanabe *et al.*, 1992. The crossed hatched, open, dotted and slashed bars represent the *cspA* promoter, its 5' untranslated region, the *cspA* coding region and the *lacZ* coding region, respectively.

The solid boxes indicate the SD sequence. The positions of deleted regions are shown with nucleotide numbers. (B) Induction patterns of various deletion constructs. At mid-log phase, cultures of *E. coli* AR137 harboring various plasmids were shifted from 37°C to 15°C.

Samples were taken at 0, 1, 2, 3, 5, 7 and 10 h after the shift and β -galactosidase activity was measured. The *cspA-lacZ* fusions: pMM67 (○); pMM022 (●); pMM023 (□); pMM024 (■); pMM025 (△); and pMM026 (▲).

Fig. 10 shows the analysis of the mRNA stability. (A) Primer extension analysis of the cells harboring the *cspA-lacZ* fusions. At mid-log phase, cultures of *E. coli* AR137 harboring various plasmids were shifted from 37°C to 15°C. For measurement of the mRNA stability at 37°C, cultures were shifted back to 37°C after 30 min incubation at 15°C and rifampicin was added to the cultures to a final concentration of 200 μ g/ml. RNAs were extracted at 0 (lane 1), 1 (lane 2), 3 (lane 3) and 5 min (lane 4) after the addition of rifampicin. For measurement of the mRNA stability at 15°C, rifampicin was added 1 h after the temperature downshift, and then RNAs were extracted at 0 (lane 5), 5 (lane 6), 10 (lane 7), and 20 min (lane 8) after the addition of rifampicin. Primer extension was carried out. (B) Graphical presentation of the results shown in (A) for 37°C and 15°C, respectively. The radioactivities of transcripts were measured using a Phosphorimager and plotted using the transcript at zero time point as 100%: pMM022 (●); pMM023 (□); pMM024 (■); pMM025 (△); and pMM026 (▲).

Fig. 11 shows the analysis of the mRNA level and translational efficiency. (A) Primer extension analysis of the *cspA-lacZ* fusions. At mid-log phase, cultures of *E. coli* AR137 harboring various plasmids were shifted from 37°C to 15°C. RNAs were prepared from the culture at 37 °C (0 h; lane 1) and at 0.5 (lane 2), 1 (lane 3), 2 (lane 4), and 3 h (lane 5) after the temperature downshift. Primer extension was carried out as described previously (Mitta

et al., 1997). (B) Graphical presentation of the relative amounts of mRNA. Relative mRNA amounts were calculated from the radioactivities of transcripts shown in (A) using the transcript of pMM67 at 37°C as 1. The relative amount is shown on the top of each column. Column 1, 0 h; column 2, after 0.5 h; column 3, after 1 h; column 4, after 2 h; and column 5, after 3 h. (C) Relative translational efficiencies of the *cspA-lacZ* mRNAs. Translational efficiencies at 15°C were calculated by dividing the increment of β -galactosidase activity during the first 2 h after cold shock by the amount of mRNA using the following formula:

$$[(\text{Gal } 2 \text{ h}) \times (\text{OD } 2 \text{ h}) - (\text{Gal } 0 \text{ h}) \times (\text{OD } 0 \text{ h})] / (\text{avg. mRNA})$$

where (Gal 0 h) and (Gal 2 h) are β -galactosidase activities at 0 h and 2 h after temperature downshift, respectively; (OD 0 h) and (OD 2 h) are the optical densities at 600 nm of the cultures at 0 h and 2 h after temperature downshift, respectively; (avg. mRNA) is the average of relative mRNA amounts at 0.5, 1 and 2 h after temperature downshift. Relative translational efficiency of each mRNA was calculated using the efficiency of mRNA of pMM67 as 100%. Column 1, pMM67; column 2, pMM022; column 3, pMM023; column 4, pMM024; column 5, pMM025; and column 6, pMM026.

Fig. 12 shows sequence similarities of *cspA*, *cspB*, *cspG*, and *cspI* mRNAs around the SD sequence and potential base pairing between *cspA* mRNA and 16S rRNA. Nucleotide numbers of *cspA* (Tanabe *et al.*, 1992), *cspB* (Etchegaray *et al.*, 1996), *cspG* (Nakashima *et al.*, 1996), and *cspI* mRNA (Wang *et al.*, 1999) are given starting from the major transcription initiation site as +1. The sequence of 16S rRNA is from Brosius *et al.*, 1978. Nucleotides identical in the three *csp* mRNAs are shown in bold letters. The 13-base homologous sequence in *cspA*, *cspB*, *cspG*, and *cspI* are boxed (the upstream box). Positions of the SD sequence and the initiation codon are underlined. Potential base pairings between *cspA*

5' mRNA and 16S rRNA are indicated by vertical lines. Positions of RNase V1 sensitive sites (Powers *et al.*, 1988) are dotted.

Fig. 13 shows the role of the 13-base upstream box sequence in the *cspA* 5'-UTR region in the *cspA-lacZ* expression. (A) Construction of *cspA-lacZ* fusions. The constructs are drawn in the same manner as shown in Fig. 9A except for a box with wavy lines, which represents the 13-base upstream box sequence, 5'-GCCGAAAGGCACA-3' (SEQ ID NO:48) located upstream of the SD sequence. pKNJ37 is identical to pMM007 except for the deletion of the 13-base sequence. In both pMM007 and pKNJ37, *cspA* was translationally fused to *lacZ*. pKNJ38 is identical to pKM67 (Mitta, *et al.*, 1997) except for the addition of the 13-base sequence by replacing the DNA fragment between *Xba*I and *Hind*III with synthesized oligonucleotides. In both pKM67 and pKNJ38, *cspA* was transcriptionally fused to *lacZ*. (B) Cold-shock induction of β -galactosidase. The *cspA-lacZ* fusions: pMM007 (○); pKNJ37 (●); pKM67 (Δ); and pKNJ38 (▲).

Fig. 14 shows a comparison of the secondary structures of the 5'-UTRs for the deletion constructs. Secondary structures of the 5'-UTR for each deletion construct was predicted with a nucleotide sequence analysis program (DNASIS-Mac; Hitachi Software Engineering Co. Ltd.) based on the method of Zuker and Stieger, 1982. Nucleotides are numbered as the position in the *cspA* mRNA starting from the transcription initiation site as +1. The position of the deletion in each mutant is shown by an arrow with the nucleotide numbers of the deleted region. The highly conserved 13-base sequence upstream of the SD sequence designated the upstream box are boxed. The initiation codon and the SD sequence are also boxed.

Fig. 15 shows enhancement of *cspB* translation by DB. (A) *cspB*-DB-anti-DB

complementarity: the *cspB*-DB sequence is boxed and encompasses the region from codons 5 to 9 (Mitta et al., 1997). Additional *cspB* mRNA-16S rRNA possible base pairings downstream of DB are also shown. The AUG codon is circled, the SD sequence is boxed and

L-shaped arrows show the positions where the *cspB* gene was fused to *lacZ*. (B) Translational *cspB-lacZ* fusion constructs. On the top, the *E. coli cspB* gene is depicted from its 5' end. In pB3, pB13 and pB17, the *lacZ* gene is fused to *cspB* at residue +177 (3 aa), +200 (13 aa) and +212 (17 aa), respectively. The pB13sd and pB17sd are the same as pB13 and pB17, respectively, except that their SD sequences are changed from 5'-AGGA-3' to 5'-CTTC-3'.

(C) β -galactosidase activity of the *cspB-lacZ* constructs obtained before (time 0) and after (1, 2 and 3 hr) temperature shift from 37 to 15°C. *E. coli* AR137 cells were transformed with pB3, pB13, pB13sd, pB17 and pB17sd were grown in medium, and at mid-log phase ($OD_{600} = 0.4$) cultures were shifted from 37 to 15°C. β -galactosidase activity was measured. (D)

mRNA levels of pB3, pB13, pB17 or pB13sd after temperature shift from 37 to 15°C: the *cspB-lacZ* mRNAs were detected by primer extension before temperature downshift (time 0) and at 1, 2 and 3 hrs after temperature shift. (E) mRNA stability from pB3, pB13, pB17 and pB13sd: *E. coli* AR137 cells transformed with pB3, pB13, pB17 and pB13sd were grown under the same conditions described above. At mid log phase, the culture was shifted to 15°C and after 30 min., rifampicin was added to a final concentration of 0.2 mg/ml (time 0).

Total RNA was extracted at 5, 10 and 20 min. after rifampicin addition. The *cspB-lacZ* mRNAs were detected by primer extension.

Fig. 16 shows the effect of a perfectly matching DB enhancing the translation of *cspA*. (A)

Translational *cspA-lacZ* fusion constructs. The *cspA* gene structure from its 5'-end is showed at the top. pJG78DB1 and pJG78DB2 were constructed from pJG78 as described

in Experimental Procedures. The DB sequences of pJG78DB1 (12 matches) and pJG78DB2 (15 matches) are shown at the bottom.

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5 (B) β -Galactosidase activity of the *cspA-lacZ* fusion constructs after cold shock at 15°C. *E. coli* AR137 cells transformed with pJG78, pJG78DB1 or pJG78DB2 were grown in LB medium, and at mid-log phase ($OD_{600}=0.4$) cultures were shifted from 37°C to 15°C. β -galactosidase activity was measured before (time 0) and 1, 2 and 3 hr after the shift.

(C) Detection of the *cspA-lacZ* mRNAs. Total RNA from *E. coli* AR137 cells carrying pJG78, pJG78DB1 or pJG78DB2 was extracted at the same time points indicated above and used as a template for primer extension. (D) mRNA stability from the *cspA-lacZ*

10 constructs. *E. coli* AR137 cells transformed with pJG78, pJG78DB1 and pJG78DB2 were grown as described above. At mid-log phase, the cultures were shifted to 15°C and after 30 minutes rifampicin was added to a final concentration of 0.2 mg/ml (time 0). Total RNA was extracted at 5, 10 and 40 minutes after rifampicin addition. The *cspA-lacZ* mRNAs were detected by primer extension.

15 Fig. 17 shows that a perfectly matching DB enhances translation at 37°C: (A) pIN-*lacZ* constructs. The *XbaI-SaII* fragment from pJG78 or pJG78DB2 was inserted into the *XbaI-SaII* sites of pIN-III to create pINZ and pINZDB1, respectively which then were used to create pINZDB2, pINZDB3 and pINZDB4.

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20 (B) β -Galactosidase activity of the pINZ-*lacZ* constructs. Cultures of *E. coli* AR137 cells transformed with pINZ, pINZDB1, pINZDB2, pINZDB3 and pINZDB4 were grown at 37°C under the same conditions described in Figure 1. IPTG (1 mM) was added at mid-log phase

to each culture. β -Galactosidase activity was measured before (time 0) and at 0.5, 1, 2 and 3 hr after IPTG addition.

5 (C) mRNA sequences of the pINZ-*lacZ* constructs showing the position of SD, AUG and DB.

The *lacZ* in pJIG78 has a 10-match DB. The perfect match DB located after the 5th codon

has 16 residues complementary with the anti-DB. (D) shows the rate of β -galactosidase

synthesis of the pINZ-*lacZ* constructs. Cultures of *E. coli* AR137 cells carrying pINZ or

pINZDB1 were grown at 37°C under the same conditions described above. IPTG (1 mM)

was added at mid-log phase to each culture. Rate of β -galactosidase synthesis was measured

before (time 0) and 0.5, 1, 2, 3 and 4 hr after IPTG addition. Cells were pulse-labeled with

10 trans-[³⁵S]-methionine. Cell extracts from each time point were analyzed by 5% SDS-PAGE

and the β -galactosidase synthesis was measured by phosphorimager. The ratio of β -

galactosidase synthesis of pINZ and pINZDB1 is shown at each time point.

Fig. 18 shows ribosomal fractionation of *E. coli* JM83 cells transformed with pINZ or

pINZDB1. Ribosomal particles were isolated as described by Dammel and Noller, 1995.

15 Cultures of *E. coli* JM83 cells carrying pINZ or pINZDB1 were grown at 37°C in LB medium

containing 50 mg/ml of ampicillin. At mid-log phase ($OD_{600}=0.4$) 1 mM of IPTG was added

to each culture. Chloramphenicol (0.1 mg/ml) was added at 15, 30 and 60 min after IPTG

addition. The cell extracts were then layered on top of a 5-40% (w/w) sucrose gradient. The

polysomes and ribosomal subunits were separated by centrifugation at 151,000 x g for 2.5 hr

20 at 4°C. The polysome profiles were then detected by using a FPLC system. 0.2 ml from each

fraction (0.5 ml) were spotted on a Nitrocellulose membrane using the Minifold II Slot-Blot

System (Schleicher and Schuell). The *lacZ* mRNA was detected by hybridization using the

[³²P]-labeled M13-47. Phosphorimager values from the hybridization are plotted at the right

side. The pINZ and pINZDB1 mRNAs are shown in closed and open squares, respectively.

Fig. 19. shows translational enhancement by a perfectly matching DB at 37°C. **(A)**

Estimation of pINZ and pINZDB1 mRNAs. Cultures of *E. coli* JM83 carrying pINZ or pINZDB1 were grown at 37°C under the same conditions described in Figure 4. Total RNA extracted at 15, 30 and 60 min after IPTG (1 mM) addition was used as a template for primer extensions according to the procedure described previously. **(B)** β -Galactosidase activity of pINZ and pINZDB1 in multi-copy expression system. *E. coli* JM83 cells transformed with pINZ or pINZDB1 were grown at 37°C under the same condition described in Figure 20A. β -Galactosidase activity was measured before (time 0) and 0.5, 1, 1.5, 2 and 2.5 hr after IPTG (1 mM) addition (open circles and squares). Closed circles and squares represent the activities in the absence of IPTG.

Fig. 20 shows cell-free synthesis of β -galactosidase from pINZ and pINZDB1. **(A)** pINZ or pINZDB1 DNA (160 ng; 1 μ l) was added to the *E. coli* 30S extract (20 μ l) (Promega) and the transcription-translation coupled reaction was carried out. Lane 1, pINZ DNA; lane 2, pINZDB1 DNA; and lane 3, a control reaction without added DNA. Samples were precipitated with acetone and analyzed by 15% SDS-PAGE to detect the production of β -galactosidase. **(B)** Time course *in vitro* synthesis of β -galactosidase from pINZ and pINZDB1 was carried out as described above. Samples were taken after 15, 30, 60 and 120 min incubation at 37°C. **(C)** Each reaction from the time course experiment described above was done in duplicate with non radioactive methionine, spotted on nitrocellulose membrane and hybridized with [³²P] labeled M13-47 oligonucleotide.

Fig. 22 shows translational enhancement of pINZDB1 in cells with S2-depleted ribosomes **(A)** β -galactosidase activity from pINZ and pINZDB1. *E. coli* CS240 and CS239 (Shean and Gottesman, 1992) were transformed with pINZ or pINZDB1 and cultures were grown at 30°C in LB medium. At mid-log phase the cells were shifted to 42°C in the presence of 1 mM of

5 IPTG. β -Galactosidase activity was measured as Miller units before (time 0) and at 0.5, 1, 1.5, 2.5 and 3.5 hr after shift to 42°C. (B) Relative induction of the *lacZ* expression between pINZDB1 and pINZ in cells with S2-depleted ribosomes. Before the shift to 42°C (time 0) the ratio of the β -galactosidase expression from pINZ and pINZDB1 in CS239 to that of CS240 was estimated as 1, and the ratios after the shift to 42°C were calculated accordingly.

SUMMARY OF THE INVENTION

10 The invention comprises an isolated nucleic acid molecule that prolongs the expression of cold-shock inducible genes under conditions of physiological stress that elicit the cold-shock response in bacteria.

The invention further comprises an isolated nucleic acid molecule that represses the expression of cold-shock inducible genes under physiological conditions.

15 The invention further comprises an isolated nucleic acid molecule that enhances translation of cold-shock inducible genes under conditions of physiological stress that elicits a cold shock response in bacteria.

20 What has been discovered is that three of the essential processes that govern the regulation of genes that are expressed under conditions of physiological stress that elicit the cold shock response of a bacterium are mediated by the 5'-untranslated region (5'-UTR) of the mRNA transcripts encoding cold shock inducible proteins. It has been discovered that specific regions of the 5'-UTR of cold shock inducible mRNA transcripts mediate (A) the transient nature of cs gene expression after exposure of the cells the physiological stress that induces the cold shock response, (B) the repression of cs gene expression at physiological temperature, and (C) enhancement of the translation of cold shock inducible mRNA transcripts by the ribosome.

It has also been discovered that DNA constructs encoding these regulatory elements, either alone or in combination with themselves, or with other regulatory sequences known in the art, e.g., promoters, downstream boxes and transcriptional terminator sequences, can be exploited to prolong the expression and increase the efficiency of translation of cs genes or of a heterologous gene under conditions of physiological stress that induce the cold shock response of a bacterium, and repress the expression of said genes under normal physiological condition. This is advantageous for the hyper-expression of a desired protein product which may be unstable at physiological temperatures, or may fold improperly and exist in inclusion bodies within the host cell at physiological temperatures, or may be degraded by the host cell.

The invention offers a further advantageous trait in that the translation of normal cellular proteins is inhibited under the conditions where the desired protein product is being hyper-expressed. This allows the desired product to accumulate in the host cell and simplifies the purification of the desired product from host cell polypeptides.

Accordingly, the invention also comprises transformed bacteria carrying the vectors which contain one or more of the above described elements and transformed bacteria carrying vectors with one or more of the above described elements and a target gene sequence for expression. Such target sequence can be a cold-shock gene sequence or a heterologous gene sequence.

DETAILED DESCRIPTION OF THE INVENTION

As reported by Sprengart et al., the downstream box (DB) of bacteria plays an important role in the translation of mRNA to produce proteins. The DB binds to a portion of the bacterial 16S rRNA near the 3' end and is thought to help position the mRNA and rRNA in proper relative position for translation to occur.

It has been discovered that during the time when the ADB is annealed to the DB of an overexpressed mRNA, the 16S rRNA is not capable of participation in the translation of cellular mRNAs other than the annealed overexpressed mRNA. It has been further discovered that the entire protein-making machinery of a bacterium may be shut down by providing to the bacterium an mRNA, which encodes a DB which is substantially complementary to the ADB of the 16S rRNA, which anneals to all or substantially all of the bacterial 16S rRNA.

The term "complementary" is used herein, it is intended to include "substantially complementary". Thus, the term "complementarity" does not require perfect complementarity. It is sufficient that the two sequences be "complementary" as defined in Kahl, Dictionary of Gene Technology, VCH Publishers, Inc. (1995), which is incorporated herein by reference. That is, two nucleotide sequences are complementary if they are capable of forming a hydrogen-bonded duplex with each other according to Watson-Crick base-pairing rules. Two complementary RNA sequences, or an RNA and a DNA sequence, will form pairings of A-U, G-C, or G-U. "Complete complementarity" is not required.

"Homologous" as used herein refers to molecules which have substantially the same molecular sequence of the referenced nucleic acid sequence, but may contain additions, deletions, or substitutions. Homologous molecules are defined as those molecules which hybridize under low or high stringency conditions to a nucleic acid molecule that is precisely complementary to the referenced nucleic acid molecule and which performs the same function as the referenced nucleic acid.

By way of example, and not limitation, low stringency conditions for hybridization are: Filters containing DNA are pretreated for 6 hours at 40°C in a solution of 35% formamide, 5xSSC, 50 mM Tris-HCL (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1%

BSA, and 500 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and $5-20 \times 10^6$ cpm ^{32}P -labeled probe. Filters are then incubated in hybridization mixture for 18-20 hours at 40°C, and washed for 1.5 hours at 55°C in a solution containing 2xSSC, 25 mM Tris-HCL (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh wash solution and incubated for an additional 1.5 hours at 60°C. The filters are blotted dry and exposed for autoradiography. If desired, or if required, a third wash step may be conducted for 1.5 hours at a temperature of 65-68°C and the filters can be reexposed to film.

For example, and not by way of limitation, high stringency conditions may be as follows: Prehybridization of filters containing the nucleic acid to be probed is carried out for 8 hours to overnight at 65°C in buffer containing 6xSSC, 50 mM Tris-HCL (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA and 500 µg/ml denatured salmon sperm DNA. The filters are hybridized for 48 hours at 65°C in a prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and $5-20 \times 10^6$ ^{32}P -labeled probe. Washing of filters is performed for 1 hour in a solution of 2xSSC, 0.01 PVP, 0.01% Ficoll, and 0.01% BSA at 37°C, followed by a wash for 45 minutes in 0.1xSSC at 50°C. The filters are blotted dry and exposed for autoradiography. Other protocols for high and low stringency hybridization known in the art may be substituted.

As is known, the ADB is a nucleotide sequence of about 14 bases which is positioned in the 3' end of the 16S rRNA, in close proximity to the decoding region of 16S rRNA. The 16S rRNA nucleotide sequence of known bacteria is known and can be found in the GenBank database. Thus, for a selected bacterium, the ADB can be readily identified by comparison to the sequence of the ADB in a bacterium in which the sequence is known, for example *E. coli*. Once the ADB is identified, a DB complementary to the ADB can be constructed, and incorporated into an appropriate mRNA, as described below.

The mRNA of the invention is an isolated mRNA or an mRNA which has been transcribed from an isolated DNA. The mRNA comprises an initiation codon, which codon is preferably AUG. Other suitable initiation codons for the mRNA include GUG and UUG.

The mRNA of the invention further comprises a downstream box sequence, which is typically 3' to the initiation codon. The codons of the DB may or may not be in-frame with the initiation codon. The DB sequence may be immediately adjacent to the initiation codon so that there are no intervening nucleotides. Generally, the DB is separated from the initiation codon by an intervening nucleotide sequence between 1 and 30 nucleotides long. The base sequence of the intervening sequence is immaterial and may be constituted of any sequence of nucleotides. Preferably, the intervening nucleotide sequence is 9 to 15 nucleotides in length, with a most preferred length of 12 nucleotides. Alternatively, the DB may overlap the initiation codon. That is, any one of the three nucleotides of the initiation codon of the mRNA of the invention may form the 5' end of the DB.

The DB sequence of the mRNA of the invention is a nucleotide sequence which is complementary to the ADB of the 16S rRNA of a bacterium. Generally, the DB is between 6 and 20 bases long, preferably between 8 and 14 bases long, although the DB may be longer than 20 bases. For example, the DB may comprise nucleotides which are complementary to nucleotides 3' or 5', or both, to the ADB. Regardless of length of the DB, a higher degree of complementarity between the DB and the ADB is associated with more effective annealing, resulting in more efficient inhibition of bacterial protein synthesis, in accordance with the method of the invention.

In addition to the initiation codon, the DB, and any intervening sequence, the mRNA construct of the invention may comprise a nucleotide sequence 5' to the initiation codon or 3' to the DB. For example, the mRNA construct may comprise a sequence 3' to the DB which

encodes a polypeptide or may comprise a termination codon. Likewise, the mRNA construct may comprise an untranslated sequence and/or a Shine-Dalgarno sequence 5' to the initiation codon.

The length of the mRNA construct, including the initiation codon, any intervening sequence, and DB, and exclusive of any additional nucleotides at the 5' or 3' end, may be any length between 8 nucleotides to about 45 nucleotides. Of course, if the mRNA comprises a 5' or 3' sequence in addition to the above essential components, such as a Shine-Dalgarno sequence, the mRNA may be much longer, up to several hundreds of nucleotides in length.

Preferably, although not necessarily, the mRNA construct is free of sites for RNA endonucleases. It is especially preferred that the portion of the mRNA construct comprising the essential portions of the construct, that is the initiation codon and the DB, be free of sites for RNA endonucleases, which might otherwise degrade the mRNA construct and free the bacterial 16S rRNA to bind to bacterial mRNAs.

The mRNA construct of the invention may have a sequence which is similar or identical to an mRNA sequence found naturally in a bacterium. For example, the mRNAs for several cold-shock proteins, such as the mRNAs for *E. coli* proteins CspA, CspB, CspG, CsdA, and RbfA, comprise a Shine-Dalgarno sequence, an initiation codon, and a downstream box substantially complementary to the ADB of the *E. coli* 16S rRNA. Other *E. coli* mRNAs which contain a Shine-Dalgarno sequence, an initiation codon, and a downstream box complementary to the *E. coli* ADB include RecA, Hns, NusA, InfB, and CspD.

Below are several non-limiting examples of suitable DBs for the mRNA construct. Each of the following DB is substantially complementary to the ADB of the *E. coli* 16S rRNA which ADB has the sequence:

ADB 3' (-1481) UACUUAGUGUUUCA (-1469) 5' (SEQ ID NO:1)

DB #1: 5' AUGACUGGUAUCGU 3' (SEQ ID NO:2)

DB #2: 5' AUGACUGGUUUCGU 3' (SEQ ID NO:3)

DB #3: 5' AUGACUGGUUUAGU 3' (SEQ ID NO:4)

5 DB #4: 5' AUGAGUUAUGUAGA 3' (SEQ ID NO:5)

DB #5: 5' AUGGCGAAAAGAAU 3' (SEQ ID NO:6)

A suitable mRNA construct according to the invention can be constructed using any one of the above DBs, or other suitable DB, for example:

5' AUGX_(n)AUGACUGGUAUCGU 3' (SEQ ID NO:7)

10 where n is a whole number from 0 to 30, and X is G, C, U, or A, wherein each occurrence of X may be the same as or different from any other occurrence of X.

Alternatively, the 5' end of the DB overlaps the initiation codon.

The DNA of the invention is any isolated DNA which encodes for a mRNA which is suitable for the mRNA construct of the invention, as described above. The DNA may further
15 comprise an additional nucleotide sequence 5' to the initiation codon, which sequence may include a promoter sequence. Such promoter sequences may be used to control transcription of the mRNA construct. The DNA may comprise a sequence 5' to the initiation codon which sequence has a function other than as a promoter, such as a Shine-Dalgarno sequence, and/or a sequence which has no known function. The DNA may comprise a sequence 3' to the
20 portion encoding the DB of the mRNA construct, which sequence may include, for example, a termination codon, or may encode a polypeptide, and a sequence required for transcription termination.

An example of a suitable DNA which encodes for the mRNA construct of the invention is: 5' ATGY_(n)ATGACTGGTATCGT 3' (SEQ ID NO:8) where n is a whole number

from 0 to 30, and Y is G, C, T, or A, wherein each occurrence of Y may be the same as or different from any other occurrence of Y. Alternatively, the 5' end of the DB overlaps the initiation codon, ATG. The DNA may contain additional sequences, as stated above, at the 5' and/or 3' end of the DNA.

5 The DNA sequence of the invention may be contained within a vehicle or cloning vector, such as in a plasmid or phage vector. The DNA sequence in the vector may be under the control of a promoter sequence located 5' to the initiation codon. These vectors containing the DNA of the invention may be used to transform a host bacterium which may be used to overexpress the mRNA of the invention, that is to produce the mRNA in the
10 bacterium at levels higher than produced in similar non-transformed bacteria. Any bacterium which may be transformed by means of a cloning vector is a suitable host for the DNA sequence of the invention. Methods of producing cloning vectors and transforming bacteria are known in the art and are taught, for example, in Ausubel et al., Current Protocols in Molecular Biology, J. Wiley & Sons, Inc. (1995), which is incorporated herein by reference.

15 Overexpression of the mRNA sequence of the invention results in the production of the mRNA in an amount which is higher than that found normally in the bacteria. To whatever extent the mRNA is overexpressed, the production of bacterial proteins is inhibited. If the mRNA is expressed at a high enough level, production of bacterial proteins will be completely stopped, which may lead ultimately to death of the bacterium.

20 Therefore, the construct producing the mRNA is useful as an antibiotic to kill or to stop the growth of bacteria. The construct producing the mRNA may be packaged in a bacteriophage which would permit the mRNA to be used as a disinfectant or as a topical antibiotic preparation. It is conceivable that strategies for delivery will be devised to permit transformation of bacteria which are causing infection of a plant or animal, such as a mammal

like humans, dogs, cats, cattle, horses, and livestock. Such antibiotics are safe for use in eukaryotes, as eukaryotes lack the 16S rRNA that is present in bacteria.

According to the method of the invention, a mRNA comprising an initiation codon and a DB which is complementary to the ADB of the 16S rRNA of a bacterium, is caused to be overexpressed in a bacterium, and is then allowed to anneal to the ADB of the 16S rRNA of the bacterium, thereby inhibiting production of proteins encoded by other mRNAs in the bacterium.

Any means of delivery which results in overexpression of the mRNA of the invention is suitable for the method of the invention. For example, the bacterium may be transformed by means of a vehicle harboring a DNA sequence which codes for the mRNA of the invention.

If desired, expression of the mRNA sequence of the invention is controlled by placing the DNA sequence under the control of an inducible promoter. For example, if it is desired to kill a harmful bacterium or block its growth while sparing a beneficial bacterium, the DNA sequence may be placed under the control of a promoter which is responsive to a product which is present only in the first bacterium. In this way, the lethal antibiotic effect of the mRNA of the invention will affect only the undesirable, harmful bacterium.

Another means of controlling the expression of the protein production-inhibiting mRNA sequence is to employ a DNA sequence which codes for an mRNA which is unstable under certain conditions.

For example, the 5' untranslated region (5' UTR) of the mRNA of the *E. coli* cold-shock protein, CspA, contains a region immediately 5' to the Shine-Dalgarno region which is susceptible to degradation, presumably by RNase E, at physiological growth temperatures of about 37°C. Therefore, the *cspA* mRNA containing the 5' UTR is unstable under normal

growth conditions, having a half life estimated to be approximately 12 seconds. Other cold-shock proteins, such as *E. coli* CspB and CsdA, are similarly unstable at physiological growth temperatures due to instability of their mRNAs. Upon cold shock, such as when the temperature is reduced to 15°C, the half life of the *cspA* mRNA increases dramatically, to about 15 minutes, an increase in stability of about 75 times over the mRNA at normal physiological growth temperatures.

Because of the instability at 37°C of an mRNA containing the 5' UTR of *cspA* mRNA, this region, or the 5' UTR of the *cspB* or *csdA* mRNA, can be used to control the expression of the mRNA sequence of the invention, so that its antibiotic effect occurs only below physiological growth temperatures, such as under cold-shock conditions. The antibiotic effect of the method of the invention is augmented at cold-shock conditions because a cold-shocked bacterium requires new ribosomal factors, whose synthesis is blocked by overproduction of an mRNA containing the DB sequence.

The antibiotic effect of the method of the invention in which the mRNA of the invention is caused to be overexpressed within a bacterium is increased concomitantly with an increase in copy number of the mRNA which is to be expressed. That is, whereas a minimal overexpression of the mRNA of the invention will inhibit the production of proteins by the bacterium, such an inhibition may not be sufficient to prevent further growth of the bacterium or to kill the bacterium. Higher levels of expression of the mRNA result are positively correlated with increased inhibition of protein production. When the copy number is sufficiently high in the bacterium, protein production will be completely blocked.

A similar effect is noted with respect to complementarity of the DB of the overexpressed mRNA and the ADB of the bacterial 16S rRNA. Overexpression of an mRNA comprising a DB with 100% complementarity will be more efficient in binding to the ADB

than will be an mRNA comprising a DB with lesser (75%) complementarity. Thus, the protein blocking effect of an mRNA having a more highly complementary DB will be more pronounced compared to that of an mRNA having a less complementary DB. Therefore, when using an mRNA having a less complementary DB, it may be useful to express the mRNA in a higher copy number to achieve the same or similar antibiotic results as with an mRNA having a more complementary DB.

The translational inhibitory properties of the downstream box are also advantageous for overexpressing a heterologous gene in a transformed bacterium after cold shock.

Inhibition of the translation of endogenous bacterial proteins will allow the heterologous gene product to accumulate to very high levels in the transformed organism. Furthermore, a construct containing the downstream box in conjunction with a strong promoter and the 5' untranslated region of a cold shock inducible gene, which functions to stabilize the mRNA transcript at reduced temperature, will direct efficient high level expression of the heterologous gene at reduced temperature.

Another important embodiment of the invention relates to the role of the 5' -end untranslated region of the mRNA for *cspA*, the major cold-shock protein of *E. coli*, in cold shock adaption.

When the culture temperature of exponentially growing *E. coli* cells are shifted from 37 to 10°C, there is a growth lag period before reinitiation of cell growth (Jones et al. 1987). Similar to the heat-shock response, *E. coli* responds to the temperature downshift by inducing a specific pattern of gene expression called cold-shock response, which includes induction of a set of proteins defined as cold-shock proteins (Jones et al. 1992; for review, see Jones and Inouye 1994). The cold-shock response occurs during the lag period of cell growth, and is considered to be required for cellular adaptation to low temperature.

CspA, the major cold-shock protein in *E. coli*, is dramatically induced upon temperature downshift, whose production reaches as high as 13% of total protein synthesis (Goldstein et al. 1990). Interestingly, however, CspA production during cold-shock response is transient and drops to a basal level at the time of reinitiation of cell growth at low temperature. CspA consists of 70 amino acid residues, and shows 43% identity to the "cold-shock domain" of the eukaryotic Y-box protein family which is known to be associated with gene regulation and mRNA masking (for review, see Wolffe et al. 1992; Wolffe 1993). The three-dimensional structure of CspA has been determined, consisting of five anti-parallel β -sheets which form a β -barrel structure (Newkirk et al. 1994; Schindelin et al. 1994). Two RNA binding motifs, RNP1 and RNP2, are identified on β 2 and β 3 sheets, respectively. In the structure, seven out of eight aromatic residues are located on the same surface and a single-stranded DNA was shown to interact with these surface aromatic residues (Newkirk et al. 1994). It has been proposed that CspA function as an RNA chaperone to facilitate translation efficiency at low temperature (for review, see Jones and Inouye 1994).

Cold Box

In one embodiment of the invention, a DNA sequence is capable of prolonging the normally transient expression of the cold shock genes during the adaptation of a bacterium to physiological stress that elicits the cold shock response. Thus, the transient nature of the cold shock response and the normally transient expression of the cold shock inducible gene is blocked and expression is continued at high levels for an extended period of time, such as at least 2 to 3 hours. The DNA sequence that is competent to confer this activity comprises the 5'-UTR or at least a portion of the 5'UTR of a cold shock inducible mRNA transcript and a promoter, active under conditions of physiological stress that induce the cold shock response

in a bacterium. Furthermore, it was found that not the entire 5'-UTR was essential, but that the sequence responsible for blocking the transient expression of the cold shock genes, like *cspA*, resides within the first twenty five nucleotides of the 5'-UTR.

Comparison of this nucleotide sequence which is competent for this activity with other cold shock genes showed that *cspB*, *cspG* and *csdA* possessed similar sequences within their respective 5'-UTR and were expressed in a transient manner in response to physiological stress that induces the cold shock response. This suggests that these genes are regulated in a similar manner to *cspA*. Based upon these sequence comparisons, the sequence responsible for this activity, hereafter designated the cold box, was shown to be situated between nucleotides +1 and +11 of the 5'-UTR of *cspA*, *cspB*, *cspG* and *csdA*.

The cold box normally functions to down regulate the expression of cold shock genes. This was shown by hyper-expression of just the *cspA* 5'-UTR in *E. coli*, which resulted in a prolongation of the expression of cold shock inducible genes in these cells. The cold box functions probably by interacting with the CspA protein itself, or with another protein whose function is dependent upon CspA. This was shown by subsequently hyper-expression of the CspA protein in the *E. coli* cells described above and observing a suppression of the prolonged expression. Therefore, the cold box appears to comprise a repressor binding site that functions to repress the expression of cold shock genes after their induction, resulting in transient expression of these genes. As the levels of CspA protein increase after induction of its gene, the CspA begins to interact with the cold box, either directly by binding the cold box sequence or indirectly by stimulating another factor to interact with the cold box, resulting in repression of the cold shock inducible genes.

Repressor of cold shock gene expression at physiological temperatures

The 5' UTR is unique in that it is longer than most *E. coli* 5'UTRs. It plays multiple functions in *cspA* expression. It represses *cspA* expression at 37°C. These different activities have been mapped by Yamanaka et al (unpublished manuscript) by constructing and analyzing defined deletions through the 5'-UTR region. The sequences mediating repression of *cspA* expression at 37°C reside between +56 and +117 of the 5'-UTR of *cspA* (see Yamanaka et al., unpublished manuscript). It is likely that optimal repression requires other sequences in the 5'-UTR as well. In other words, the effect of deleting the +56 to +117 region was significant, but did not account for the entire level of repression observed with the intact 5'-UTR. Thus, his conclusion that other sequences may also play a role in repressing *cspA* expression at 37°C. However, the localization of at least a portion of the repression activity to a specific region of the 5'-UTR is novel. The 5'-UTR is starting to be dissected into functional domains and the +56 to +117 region represents a new functional region.

The UTR also has a positive effect on mRNA stability at 15°C. This was determined by noting an increase in steady state levels of mRNA of constructs having the UTR. However, even though more mRNA was present in these experiments, the mRNA was not translated unless the DB was present.

The effects of increasing mRNA stability and increasing mRNA translatability are thus mediated by independent sequences of the *cspA* transcript. However, the specific sequences that mediate mRNA stability are not well characterized in this region.

Translational enhancement of cold shock mRNA

The 5'-UTR enhances the translatability of *cspA* transcripts through a sequence situated between +117 and +143 of the 5' UTR. Comparison of this region between different cold shock inducible revealed a 13 base sequence (+123 - +135) having the sequence 5'-

GCCGAAAGGCACA-3' (SEQ ID NO:48) that was conserved among *cspA*, *cspB*, and *cspG* and may represent enhancer of translation. Thus, this sequence mediates efficient translation of the mRNA that possesses it. This 13 base region exhibits homology with the 16S rRNA, similar to what was previously observed with the DB, but the new translational enhancer region is complementary to a different region of the 16S rRNA than DB. Therefore, this region of the 5'-UTR may also assist in translating *csp* mRNAs by a mechanism similar to the downstream box interactions with ribosomal RNA.

In another embodiment of the invention, expression plasmids, capable of high level expression of cold shock inducible genes, or of a heterologous gene, are constructed. Such expression plasmids contain DNA fragments encoding the 5'UTR or a portion or portions thereof of a cold shock inducible gene, comprising one or more of the regulatory elements described above, positioned downstream of a promoter that is functional under conditions of physiological stress that induce the cold shock response in a bacterium. Such promoters may be selected from the group including the *cspA*, *cspB*, *cspG* or *csdA* promoters, the *E. coli lpp* promoter, or any other such promoter that is active under conditions of physiological stress. Such expression plasmids may also contain downstream of the promoter and DNA fragment encoding the 5'-UTR comprising one or more of the regulatory elements described, and a transcriptional terminator sequence. Examples of transcriptional terminator sequences are well known in the art and include sequences such as the *E. coli rrnB* terminator.

The expression plasmids of the invention may also comprise a restriction site, or a sequence comprising multiple restriction sites, such site or sites situated between the 5'-UTR encoding one or more of the regulatory elements and the transcriptional terminator to facilitate the insertion of a heterologous gene.

Finally, the expression plasmids of the invention may comprise additional sequences

known in the art to facilitate the efficient translation of the expressed gene. Such sequences may include a Shine-Dalgarno sequence, situated between the 5'UTR sequence and the restriction site(s) and/or a DNA fragment encoding a downstream box, situated between the Shine-Dalgarno sequence and the restriction site(s). The source of the Shine-Dalgarno sequence is not especially limited, and may be derived from cold shock proteins or may be from another gene. Such expression plasmids are capable of directing high level expression of a heterologous gene for a prolonged period of time under conditions of physiological stress that elicit the cold shock response of a bacterium. Under these conditions, the synthesis of endogenous proteins by the host bacterium is blocked, allowing the product of the heterologous gene to accumulate to high levels within the cell.

In a preferred embodiment of the invention, the expression vector comprises a promoter, a 5'UTR, cold box, Shine-Dalgarno sequence and a downstream box (DB). This expression vector may be used so that a nucleic acid molecule encoding a target protein may be ligated into the expression vector using at least one restriction site.

In another embodiment of the invention, nucleic acid molecules encoding proteins that are unstable, or that fold improperly in the bacterial host cell at physiological temperatures can be expressed at temperatures below the physiological temperature of the host bacterium. Such nucleic acid molecules may be inserted, preferably in-frame to the restriction site or sites to allow transcription of the nucleic acid and translation of the resulting mRNA. Such conditions may facilitate the proper folding, increase the stability or decrease the rate of degradation of the expressed product.

The plasmids or vectors may be used to transform bacteria by any method known in the art, including, but not limited to calcium chloride transformation, electroporation, and the like. The bacterial species which may be transformed is not particularly limited. In a

preferred embodiment, *E. coli* is used. The transformed bacteria may be used to overexpress a target protein of interest, or may be used to produce large amounts of the plasmids or vectors for subsequent isolation and purification.

In a preferred embodiment for overexpression of a target protein, a nucleic acid molecule encoding a target protein is ligated into the plasmid using one or more restriction sites, preferably in-frame to the initiation codon, if an initiation codon is provided upstream of the insertion site for the target protein. Alternatively, if the nucleic acid molecule encoding the target protein has its own initiation codon, the nucleic acid encoding the target protein may be ligated into the plasmid such that its own initiation codon will serve as the initiation codon in the transcript.

The constructs may be designed and assembled to include a selectable marker. That is, for example, a drug resistance gene which allows transformed bacteria to grow in the presence of a drug which does not permit the growth of non-transformed bacteria. Any selectable marker known in the art may be used. Examples of selectable markers include, but are not limited to ampicillin resistance, neomycin resistance, kanamycin resistance and tetracycline resistance.

The constructs may also include an inducible promoter. The inducible promoter may direct the synthesis of the target protein upon induction. For example, and not by way of limitation, a lacZ promoter may be included which may be induced by the addition of IPTG to the culture medium.

In a preferred embodiment, the constructs will contain a promoter which is active under conditions that elicit a cold-shock response in bacteria, such as by shifting the temperature of the culture medium containing the transformed bacteria to 10-15°C. Preferably, overexpression of a target protein in conditions that elicit a cold-shock response

reduces the synthesis of at least one native bacterial protein. More preferably, the synthesis of many native proteins is reduced or blocked.

The following examples are provided to illustrate aspects of the invention and are not to be construed in any way as limiting the scope of the invention, which is defined in the appended claims.

EXAMPLES

Example 1: Construction of Plasmids

pJJG02 was constructed from pJJG01 (Goldstein *et al.*, 1990) as follows: A 998-bp fragment which contains the entire *cspA* gene was obtained from pJJG01 by *Hind*III and *Xmn*I digestion. This fragment was then treated with the Klenow fragment of DNA polymerase (Life Technologies), and inserted into the *Sma*I site of pUC9.

pJJG21 was constructed from pJJG02 by creating an *Xba*I site immediately upstream of the Shine-Dalgarno sequence of *cspA* as follows:
+13AATTT(A)C(T)TAG(A)AGGTAA+153 (SEQ ID NO:9)(the original nucleotides in the parentheses were substituted by the underlined nucleotides; ref. 1). pJJG81 was constructed from pJJG02 by creating an *Xba*I site immediately downstream of the transcription initiation site of *cspA* as follows: +1ACGGTTCTAGACGTA+15 (SEQ ID NO:10)(nucleotides underlined represent the inserted bases).

pJJG78 is a transcriptional fusion of the 0.6-kb *cspA* upstream region and *lacZ* as follows: the 1-kb *Eco*RI/*Bam*HI fragment containing *cspA* from pJJG21 was filled in with Klenow enzyme and ligated into the *Sma*I site of pUC19. Then, the 0.6-kb *Xba*I fragment containing the *cspA* regulatory region (from -457 to +143) was excised and ligated into the *Xba*I site in pKM005 (Inouye, M. *et al.*, 1983) in the correct orientation.

pUC19-600 was constructed by insertion of the 0.6-kb *EcoRI/XbaI* fragment from pJJG21 into the *EcoRI/XbaI* sites of pUC19. pJJG81/X,S containing fragment 1 (Figure 3) was constructed by removing the 0.74-kb *XbaI/SalI* fragment from pJJG81. Both ends were treated with Klenow fragment, followed by self-ligation. All the other constructs shown in Fig. 3 were made by PCR (Boehringer Mannheim protocol). PCR amplified fragments were inserted into the *SmaI* site of pUC19. All PCR products were confirmed by DNA sequencing (Sanger *et al.*, 1977).

p2JTEK was constructed as follows: PCR product by primer 3549 5'-CGGCATTAAGTAAGCAGTTG-3' (SEQ ID NO:11) and primer 4428 5'-CTGGATCCTTTAATGGTCTGTACGTCAAACCGT-3' (SEQ ID NO:12) was cloned into the *SmaI* site of pUC19. This PCR product contains *cspA* from -146 to +25 as the *cspA* transcription start site is defined as +1. Then the transcriptional terminator of *cspA* was amplified by PCR using primer 6290 5'-CGGAATTCAGCCTGTAATCTCT-3' (SEQ ID NO:13) and 4860 5'-CTGTCGACTTACTTACGGCGTTGC-3' (SEQ ID NO:14). The PCR product was then digested with *EcoRI* then cloned into the plasmid described above which was digested with *EcoRI* and *SspI*. The 52-bp *KpnI* and *EcoRI* fragment from Bluescript II SK was then cloned into the *EcoRI* and *KpnI* site. All PCR products were confirmed by DNA sequencing (Sanger *et al.*, 1977).

p6mTEK was constructed in the same way as p2JTEK except that the first PCR was carried out with different primers: primer 3552 5'-GACAGGATTAAAAATCGAG-3' (SEQ ID NO:15) and 6196 5'-AACCGTTGATGTGCA-3' (SEQ ID NO:16). This PCR product encompasses *cspA* from -278 to +6 as the *cspA* transcription start site is defined as +1. All PCR products were confirmed by DNA sequencing (Sanger *et al.*, 1977).

The pulse-labeling experiments were carried out as described previously (Jiang *et al.*,

1993). Proteins were analyzed either by polyacrylamide SDS-gel electrophoresis (Inouye S. *et al.*, 1982) or by two-dimensional electrophoresis as described previously (Jones *et al.*, 1987).

Each 5'-UTR deletion was introduced by two step PCR. For the first step, two PCRs were carried out for each mutation. One reaction was done with a combination of primer 67F, 5'-ccttgctagCCGATTAATCATAAATATG-3' (SEQ ID NO:17)(nucleotides -67 to -49 of *cspA*), and mutation primer R, which contains desired mutated sequence and is complimentary to *cspA*, and the other was done with primer #4311, 5'-ccggatccagGTTGAACCATTTT-3' (SEQ ID NO:18) (complementary to +186 to +198) and mutation primer F, which also contains the same mutation and is same direction to *cspA*. The lower cases are extra nucleotides to create *NheI* or *BamHI* site (underlined) and the numbers are given using major transcription initiation site at +1, which was determined by Tanabe *et al.* (1992). For construction of pMM022, pMM023, pMM024, pMM025, and pMM026, primer D1R, 5'-ACTACACT/TTGATGTGCATTAGC-3' (SEQ ID NO:19)(complementary to -15 to +1 / +28 to +35), primer D2R, 5'-CAACGATAA/GCTTTAATGGTCTGT-3' (SEQ ID NO:20) (complementary to +13 to +27 / +56 to +64), primer D3R, 5'-TAAAGG/CTCTTGAAGGGACTT-3' (SEQ ID NO:21)(complementary to +41 to +55 / +86 to +91), primer D4R, 5'-CGGCGATAT/AATGTGCACTACGAGGG-3' (SEQ ID NO:22) (complementary to +69 to +85 / +118 to +126), and primer D5R, 5'-TACCTTTAA/GGCGTGCTTTACAGATT-3' (SEQ ID NO:23)(complementary to +101 to +117 / +144 to +152) was used as the mutation primer R and primer D1F, 5'-GCACATCAA/AGTGTAGTAAGGCAA-3' (SEQ ID NO:24)(nucleotide -8 to +1 / +28 to +42), primer D2F, 5'-TAAAGC/TTATCGTTGATACCC-3' (SEQ ID NO:25)(nucleotide +22 to +27 / +56 to +70), primer D3F, 5'-TCAAGAG/CCTTTAACGCTTCAAAA-3' (SEQ ID NO:26)(nucleotide +49 to +55 / +86 to +102), primer D4F,

5'-GCACATT/ATATCGCCGAAAGGC-3' (SEQ ID NO:27)(nucleotide +79 to +85 / +118 to +132), and primer D5F, 5'-AAAGCACGCC/TTAAAGGTAATACACT-3' (SEQ ID NO:28)(nucleotide +108 to +117 / +144 to +159), was used as the mutation primer F, respectively, where the position of each deletion is indicated by a slash. A plasmid, pJIG02 (Goldstein et al. (1990), which contains the wild-type *cspA*, was used as template DNA. Then, each set of the first PCR products were mixed, heat denatured, annealed, and extended by Taq polymerase. The resulting products were further amplified by PCR using primer 67F and primer #4311. The final PCR products were digested with *NheI* and *BamHI*, and inserted into the *XbaI*-*BamHI* site of pKM005 (Inouye 1983). For pKNJ37, the PCR fragment was cloned into the *XbaI*-*BamHI* site of pRS414X, a pRS414 derivative (Simons *et al.*, 1987), in which the unique *SmaI* site has been changed to an *XbaI* site.

For the construction of pMM007, PCR was carried out using primer 67F and primer #4311 as primers and pJIG02 as a template. The PCR fragment was digested with *NheI* and *BamHI*, and inserted into the *XbaI*-*BamHI* site of pRS414X.

pKNJ38 was constructed as follows: oligonucleotide #8509, 5'-CTAGCCGAAAGGCACAAATTAAGAGGGTATTAATAATGAAAGGGGGAATTCCA-3' (SEQ ID NO:29), and oligonucleotide #8510, 5'-AGCTTGGAATTCCCCCTTTCATTATTAATACCCTCTTAATTTGTGCCTTTCGG-3' (SEQ ID NO:30) were first annealed and then cloned into pKM67 (Mitta *et al.*, 1997) digested with *XbaI* and *HindIII*.

The DNA sequences of all the constructs were confirmed by DNA sequencing using the chain-termination method (Sanger *et al.*, 1977).

E. coli AR137 harboring different plasmids was grown at 37°C to mid-log phase in 15

ml of M9-Casamino acid medium using a 125-ml flask. The culture was then transferred to a 15°C shaking water bath. Culture temperature reached 15°C from 37°C within 2 to 3 min under the condition used. A 1.5-ml culture was taken immediately before the temperature downshift (0hr), and at 1, 2, 3, 5, 7, and 10 hr after the temperature downshift.

- 5 β-galactosidase activity of the culture was measured according to Miller (1972). The assay was done in duplicate at each time points.

cspB DNA fragments were amplified by PCR using synthetic oligonucleotide primers containing the *Bam*HI site at the 5' end. A plasmid, pSJ7 (Lee *et al.*, 1994) carrying the wild-type *cspB* gene was used as a template DNA to create the PCR fragments B3, B13 and B17.

- 10 The 5'-end oligonucleotide primer used in each of the above PCR reactions is 5'-CCGGATCCAGCTTTAATATAGCT-3' (SEQ ID NO:31). The 3'-end oligonucleotide primers for the PCR products B3, B13, and B17 were 5'-CCGGATCCAGATTTGACATTCTACA-3' (SEQ ID NO:32), 5'-CCGGATCCAGGTAAACCATTTT-3' (SEQ ID NO:33), and
- 15 5'-CCGGATCCAGACCTTTATCAGCGTT-3' (SEQ ID NO:34), respectively. A deletion of the SD sequence in the *cspB* gene was created by site-directed mutagenesis using the QuickChange™ Site Directed Mutagenesis Kit (Stratagene). The PCR reaction was carried out using the pSJ7 plasmid as a template and the oligonucleotides Bsd1 (5'-GAAAGGCTCAAGTTACTTCATGTAGAATG-3') (SEQ ID NO:35) and Bsd2 (5'-
- 20 CATTCTACATGAAGTAACTTGAGCCTTTC-3') (SEQ ID NO:36) to create pSJ7sd. pSJ7sd was used as a template to make the PCR fragments B13sd and B17sd. The 5' and 3' end oligonucleotide primers used in these PCR reactions are the same as the ones used for the PCR fragments B13 and B17. All of the above PCR products were cloned at the *Bam*HI site of pRS414 vector (Simmons *et al.*, 1987; Lee *et al.*, 1994) to create the pB3, pB13, pB13sd,

pB17, and pB17sd constructs.

The *cspA-lacZ* fusion constructs were made by the insertion of annealed oligonucleotides at the *EcoRI* site of the pJG78 (Jiang *et al.*, 1996). Annealed oligonucleotides DB1 (5'-AATTAATCACAAAGTGGG-3') (SEQ ID NO:37) with DB1' (5'-AATTCCCACCTTTGTGATT-3')(SEQ ID NO:38) or DB2 (5'-AATTATGAATCACAAAGTGGG-3') (SEQ ID NO:39) with DB2' (5'-AATTCCCACCTTTGTGATTCAT-3') (SEQ ID NO:40) were used to create pJG78DB1 or pJG78DB2 constructs, respectively.

The pIN-*lacZ* constructs were made by inserting the *XbaI-SalI* fragments from pJG78 or pJG78DB2 into the *XbaI-SalI* sites of pIN-III (Jiang *et al.*, 1996) to create pINZ and pINZDB1, respectively. Then, the annealed oligonucleotides ZDB2 (5'-CTAGCCCTT-ATTAATAATGAAAGGGGGAATTATGAATCACAAAGTGGG-3') (SEQ ID NO:41) with ZDB2' (5'-AATTCCCACCTTTGTGATTCATAATTCCCCCTTTCATTATTAATAAGGG-3') (SEQ ID NO:42) were inserted at the *XbaI-EcoRI* sites of pINZ to create pINZDB2.

Annealed oligonucleotides ZDB3 (5'-CTAGCCCTTATTAATAATGAATCACAAAGTGGG-3') (SEQ ID NO:43) with ZDB3' (5'-AATTCCCACCTTTGTGATTCATTATTAATAAGGG-3') SEQ ID NO:44) or ZDB4 (5'-CTAGAGGG-TATTAATAATGAATCACAAAGTGGG-3') (SEQ ID NO:45) with ZDB4' (5'-AATTCCCACCTTTGTG-ATTCATTATTAATACCCT-3') (SEQ ID NO:46) were inserted at the *XbaI-EcoRI* sites of pINZ to construct pINZDB3 and pINZDB4, respectively.

β -Galactosidase Activity

E. coli AR137 (*pcnB*⁻) (Harlocker *et al.*, 1993) or JM83 (*pcnB*⁻) harboring different plasmids were grown at 37°C to mid-log phase in 20 ml of LB medium containing 50 μ g/ml

of ampicillin in a 125 ml flask. The cultures were then transformed to a 15°C shaking water bath or isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. A 100 ml culture was taken at each time point. β-galactosidase activity was measured according to Miller's procedure (Miller, 1972).

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Example 2: Isolation of RNA, primer extension and ribosome analysis

E. coli AR137 harboring different plasmids was grown under the same conditions used for the β-galactosidase assay described above. In order to estimate the amount of *cspA-lacZ* mRNA, a 1.5-ml culture was taken at each time point and RNA was extracted by the hot-phenol method as described by Sarmientos et al., 1983.

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For the mRNA stability experiments at 15°C, rifampicin was added at 1 hr after the temperature downshift at a final concentration of 200 μg/ml to stop transcription, and a 1.5-ml culture was taken at each time point. For the mRNA stability at 37°C, the culture was first shifted to 15°C for 30 min to accumulate the mRNAs. Then, a 5-ml culture was taken and mixed with a 5 ml of the medium containing 400 μg/ml rifampicin in a glass flask kept in 37°C shaking water bath. The medium was prewarmed at 60°C. Using this method, the culture temperature immediately changed to 32°C and reached 37°C within 1 min.

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The *cspA-lacZ* mRNAs were detected by the primer extension method as described previously (Jiang et al. 1993) using a ³²P-labeled primer M13-47,

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5'-CGCCAGGGTTTCCCAGTCACGAC-3' (SEQ ID NO:47), which is complementary to a 5' -end coding sequence of *lacZ*. The products were separated on a denatured polyacrylamide gel (6%) and quantified by the use of Phosphorimager (BioRad).

Pulse Labeling

Cultures of *E. coli* AR137 (*pcnB*⁻) cells carrying pINZ or pINZDB1 were grown at 37°C under the same conditions used for the β-galactosidase assay. IPTG (1 mM) was added at mid-log phase to each culture. At each time point, 1 ml of the culture was labeled for 5 min with 100 μCi of trans-[³⁵S]methionine (1,175 Ci/mmol) (NEN Life Science Products) as described previously (Jones *et al.*, 1996). Cell extracts from each time point were loaded on a 5% SDS-PAGE and β-galactosidase synthesis was measured by phosphorimager.

Ribosome Isolation

Cultures of *E. coli* JM83 (*pcnB*⁺) cells carrying pINZ or pINZDB1 were grown in 600 ml of LB medium in a 4-liter flask under the same condition as described above. At mid-log phase IPTG (1 mM) was added to each culture. Ribosomal particles were isolated by the procedure described by Dammel and Noller (1995), with some modifications: An 100-ml aliquot from the original culture was taken at each time point and chloramphenicol was added to a final concentration of 0.1 mg/ml to stop cell growth. Cells were immediately collected by centrifugation (5,000 x g for 10 min at 4°C), resuspended in buffer 1 [20 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 100 mM NH₄Cl, 6 mM β-mercaptoethanol and 1 mg/ml lysozyme] and frozen at -80°C for a few hours. The cells were lysed by the freeze-thaw method (Ron *et al.* 1996). The cell extracts (0.5 ml) were then layered on top of a 5-40 % (w/w) sucrose gradient (7.5 ml) and the polysomes and ribosomal subunits were separated by centrifugation at 151,000 x g for 2.5 hr at 4°C using a Beckman SW-41 rotor. The polysome profiles were detected by an FPLC system and a total of 15 fractions of 0.5 ml each were collected.

Detection of the *lacZ*-mRNA

From each polysome fraction (0.5 ml) 0.2 ml was spotted on a Nitrocellulose membrane using the Minifold® II Slot-Blot System (Schleicher and Schuell). The *lacZ*-mRNA was detected by hybridization using the [³²P]-labeled M13-47 primer (S. Inouye and M. Inouye, 1991) and the amount of *lacZ*-mRNA was estimated by phosphorimager.

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***In vitro* Translation**

Using the *E. coli* S30 Extract System for Linear Templates Kit (Promega), the transcription-translation coupled reaction was carried out according to the manufacturer's protocol as follows; To 20 µl of Pre-mix containing all the amino acids except for methionine, 10 µCi of trans-[³⁵S]methionine (1,175 Ci/mmol; NEN Life Science Products) and the *E. coli* S30 extracts, 160 ng of pINZ or pINZDB1 (1 µl) was added and the mixture was incubated at 37°C for 45 min. The products were precipitated with acetone and analyzed by 15 %-SDS-PAGE.

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EXAMPLE 3: Multicopy effects of the *cspA* upstream region on cold-shock adaptation.

It has been shown that the *cspA* gene is induced immediately after the temperature downshift from 37°C to either 15 or 10°C and that the rate of CspA production reaches a peak after 1 hr at 15°C and 2 hr at 10°C after the temperature shift (Goldstein *et al.*, 1990). After this time point, CspA production sharply drops to a new basal level. The period of this transient production of CspA corresponds to the duration of growth arrest, known as the lag period, which is observed after cold shock (Jones *et al.*, 1987). Thus, such a transient expression of *cspA* is considered to be required for cellular adaptation to lower temperatures.

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In order to characterize this transient expression of *cspA*, we attempted to identify the region required for the regulation of the *cspA* expression during the adaptation period. For

this purpose, pJJG78 was first constructed, in which the 600-bp *cspA* upstream region was transcriptionally fused to the *lacZ* gene (Fig. 1A). This 600-bp upstream region of *cspA* encompasses the region from -457 to +143 which is right before the Shine-Dalgarno sequence of *cspA*, as the *cspA* transcription initiation site is defined +1 (Goldstein *et al.*, 1990). *E. coli* strain CL83 was transformed with pJJG78 and the production of β -galactosidase was examined by pulse-labeling cells with [35 S]-methionine at 0, 0.5, and 3 hr after temperature downshift from 37 to 15°C. As controls, CL83 cells alone as well as CL83 cells transformed with vector pKM005 (Inouye 1983) were also used. As shown in Fig. 1B, for both CL83 and CL83/pKM005 the expression of *cspA* was highly induced at 0.5 hr after the temperature downshift (Fig. 1B, lanes 2 and 5, respectively). However, as shown previously (Goldstein *et al.*, 1990), this high expression is transient and reduced to a new basal level at 3 hr (Fig. 1B, lanes 3 and 6, respectively). Note that no *cspA* expression was detected at 0 time point (Fig. 1B, lanes 1 and 4, respectively) and that β -galactosidase was not produced at any time point for both strains (Fig. 1B, lanes 1 to 6).

In contrast to CL83 and CL83/pKM005, β -galactosidase was clearly induced in the cells with pJJG78 upon the temperature downshift (Fig. 1B, lanes 7 to 9), indicating that the 600-bp upstream region of *cspA* is sufficient for the cold-shock induction. Surprisingly, the production of *cspA* was no longer transient but remained at a high level even 3 hr after cold shock in the cells harboring pJJG78 (Fig. 1B, compare lane 9 with lanes 3 and 6). Since pJJG78 does not contain the *cspA* coding sequence, the high production of CspA at 3 hr after temperature downshift is attributed to the chromosomal *cspA* gene. It appears that under the conditions used, the chromosomal *cspA* gene failed to be repressed, in other words it became derepressed. Interestingly, there is another band indicated by X in Fig. 1B, whose expression pattern was almost identical to that of *cspA*. It is a cold-shock protein and its production was

also derepressed in the presence of pJIG78. This cold-shock protein X has been recently identified as CsdA which associates with ribosomes (Jones *et al.*, 1994).

It should also be noted that the synthesis of most cellular proteins was blocked to a larger extent in the cells harboring pJIG78 at low temperature than that in the CL83 cells and CL83/pKM005 (Fig. 1B, compare lanes 8 and 9 to lanes 2, 3, 5, and 6). These results indicate that the cellular adaptation to the low temperature is impaired with a more severe cold-shock response when cells harbor a multicopy plasmid carrying a part of the *cspA* gene.

Since the prolonged synthesis of *cspA* after cold shock was caused by pJIG78, it was hypothesized that the 600-bp *cspA* upstream region cloned in pJIG78 may sequester a factor responsible for the inhibition of *cspA* production after cold shock, resulting in the prolonged expression or the derepression of *cspA*. In order to examine this hypothesis, the 600-bp upstream region of *cspA* was re-cloned into pUC19. The plasmid is called pUC19-600. Note that the copy number of pUC19 (300 copies/cell) is about 10 times higher than pJIG78 derived from pBR322 (30 copies/cell). A pulse-labeling experiment was carried out as described previously (Jiang *et al.*, 1993). As shown in Fig. 2, in the CL83 cells, CspA production increased up to 1.5 hr and was reduced to a basal level after 3 hr at 15°C (Fig. 2, lanes 1 to 6). In CL83 cells with pJIG78, a certain level of *cspA* expression was still observed even after 24 hr at 15°C (Fig. 2, lanes 7 to 12). Patterns of *cspA* production in CL83 cells with pUC19-600 are similar to those with pJIG78 (Fig. 2, lanes 13 to 18). However, the level of the *cspA* derepression was much higher with pUC19-600 than that with pJIG78, as judged from the production of CspA at 3 and 5 hr. Thus, the higher the copy number of the *cspA* upstream region, the stronger the derepression of the *cspA* expression. Again, CsdA (indicated by X) showed the exactly same expression pattern as CspA throughout all the lanes shown in Fig. 2.

As shown in Fig. 1B, the cells with pJJG78 showed a certain inhibition of general protein synthesis at low temperature (compare lanes 8 to 11 with lanes 2 to 5, respectively in Fig. 2). Significantly, this inhibition in the cells harboring pUC19-600 was even more evident than that in the cells harboring pJJG78, in terms of both the protein synthesis rate and the inhibition time (compare lanes 14 to 17 with lanes 8 to 11, Fig. 2). The higher copy number of the 600-bp of *cspA* upstream region results in the stronger inhibition of the synthesis of other cellular proteins, indicating that cold-shock adaptation is inhibited.

EXAMPLE 4: Overproduction of the 5' untranslated region of the *cspA* mRNA.

In order to determine the precise region within the 600-bp sequence required for the derepression of *cspA* and the inhibition of cold-shock adaptation at low temperature, a series of internal fragments as shown in Fig. 3 were generated by PCR and cloned into the *Sma*I site of pUC19. Their sequences were confirmed by DNA sequencing. The ability to derepress expression of *cspA* and to inhibit cold-shock adaptation at 15°C for each construct was examined by pulse-labeling experiment. First, deletion mutations were made from the 5' end of the 600-bp fragment. As shown in Fig. 3, fragment 3 (186-base deletion), fragment 2 (312-base deletion), fragment 2E (366-base deletion) and fragment 2G (390-base deletion) all still retained the derepression function. Next, fragment 2 was further dissected into fragment 2A and 2B which overlap by 23 bp as shown in Fig. 3. Surprisingly, both 2A and 2B lost the functions. Fragment 2F which is longer by 33 bp at the 5' end than fragment 2B was also constructed, was still incapable of the functions. It was found here that the constructs which are capable of the derepression of *cspA* also result in inhibition of the cold-shock adaptation, and *vice versa*.

The fact that fragment 2 is functional for both the *cspA* derepression and the inhibition

of cold-shock adaptation, while fragment 2A is not, indicates that the *cspA* promoter region alone is not sufficient for the functions of the 600-bp fragment. Furthermore, the fact that functional fragment 2G is longer at the 5' end by 31 bp than the non-functional fragment 2F suggests a possibility that the both functions require the full *cspA* promoter for the transcription of the 5' UTR of the *cspA* mRNA. Note that the *cspA* mRNA has a 159-base untranslated sequence at the 5' end (Goldstein *et al.*, 1990). In order to confirm this possibility, the *cspA* transcripts produced from the cloned fragments (fragments 2, 2A, 2B, 2E, and 2F) were examined by primer extension. Using the total RNA fraction isolated from cells harboring various plasmids incubated for 1 hr at 15°C, primer extension was performed with two independent primers; primer 3550 which corresponds to the sequence from +124 to +143 in the 5' UTR and primer 3551 which corresponds to a part of the *cspA* coding sequence from +224 to +243. The former primer detects the *cspA* mRNA transcribed from both the plasmid and the chromosome, while the latter detects the mRNA only from the chromosomal *cspA* gene, since none of the plasmids contains the *cspA* coding region.

As shown in Fig. 4, the amounts of the transcript from the chromosomal *cspA* gene indicated by primer 3551 were basically the same among all constructs (Fig. 4, lanes 1 to 6). In contrast, the amount of the *cspA* transcripts encompassing the 5' UTR indicated by primer 3550 showed two different levels. For those nonfunctional constructs (pUC19-2A, pUC19-2B, and pUC-2F), the amounts of the transcripts detected by primer 3550 (lanes 3, 4, and 6 in Fig. 4, respectively) were almost identical to that with pUC19 (lane 1 in Fig. 4), indicating that the *cspA* regions cloned in these plasmids were not transcribed. On the other hand, for those functional constructs (pUC19-2 and pUC19-2E), much higher levels of the *cspA* transcripts detected by primer 3550 were observed (lanes 2 and 5 in Fig. 4, respectively) in comparison with the level with pUC19 (lane 1 in Fig. 4). These results demonstrate that the

5' UTR of the *cspA* mRNA was transcribed in fragment 2 and 2E, but not in fragments 2A, 2B and 2F. Therefore, the ability to prolong *cspA* expression and to inhibit the cold-shock adaptation at low temperature is clearly correlated with the transcription of the 5' UTR of the *cspA* mRNA.

5 In order to unambiguously demonstrate that the transcription of the 5' UTR of the *cspA* mRNA is required for both the *cspA* derepression and the inhibition of cold-shock adaptation, the entire promoter fragment (-457 to -1) plus 6-base (+1 to +6) region from *cspA* was cloned into pUC19. This fragment was designated fragment 1 (see Fig. 3). Thus, most of the 5' UTR of the *cspA* mRNA was deleted in fragment 1. By pulse-labeling experiment shown in Fig. 5B, fragment 1 was incapable of derepressing *cspA*, in spite of the fact that the transcripts from the *cspA* promoter were clearly detectable by primer extension (Fig. 5A). From these results, it is concluded that at least a portion of the *cspA* 5'UTR from +1 to +143 has to be transcribed to exert the effect on the *cspA* expression and the cold-shock adaptation.

15 **EXAMPLE 5: Cold-shock genes affected by the overproduction of the 5' UTR of the *cspA* mRNA.**

Next the overproduction of the 5' UTR of the *cspA* mRNA was examined to determine if the *cspA* mRNA has any effects on the expression of other cold-shock genes. The protein expression pattern of the cold-shocked cells overproducing the *cspA* 5' UTR was analyzed by two-dimensional electrophoresis. The plasmid pJJG21/X,S contains the entire *cspA* promoter and most of the 5' UTR of the *cspA* mRNA (+1 to +143), while pJJG81/X,S contains the entire *cspA* promoter but only the first 6-base region of the 5'UTR of the *cspA* mRNA. The cells harboring these plasmids were pulse-labeled as described before (Jiang *et al.*, 1993). At 37°C, the rate of protein synthesis and the protein pattern were very similar for

both strains (Fig. 6, A and B); note that no cold-shock proteins were detected. When these cells were shifted to 15°C for 1 hr (Fig. 6, C and D), the synthesis of cold-shock proteins (1) *cspA*; (2) *CspB'*; (3) *CspB*; and (4) *CsdA* became very prominent. Note that *CspB'* was co-induced with *CspB* and has been speculated to be either a modified form of *CspB* or a yet unidentified cold-shock protein (Etchegaray *et al.*, 1996). The rate of cold-shock protein synthesis for both constructs was comparable as judged from the densities of the spots. Although the synthesis of most other cellular proteins was significantly reduced for both strains compared with that at 37°C, much stronger inhibitory effects were observed in the cells transformed with pJG21/X,S. When cells were incubated at 15°C for 3 hr, synthesis of most cellular proteins recovered to a normal level with concomitant reduction of all the cold-shock proteins in the cells harboring pJG81/X,S (Fig. 6F). In contrast, for the cells harboring pJG21/X,S, the production of all the cold-shock proteins (marked by 1 to 4) was still maintained at a very high level along with reduced production of other cellular proteins (Fig. 6E). These results clearly demonstrated that overproduction of the 5' UTR of the *cspA* mRNA results in the derepression of not only *cspA* but also other cold-shock genes, suggesting that genes for cold-shock proteins are regulated by a common mechanism. It is also further confirmed that the inhibition of cold-shock adaptation is due to the overproduction of 5' UTR of the *cspA* mRNA by blocking the synthesis of other cellular proteins.

Based on the results described above, overproduction of the 5'UTR of the *cspA* mRNA causes the concomitant inhibition of other cellular proteins. This implies that cell growth upon cold shock would be more severely inhibited with the cells overproducing the 5'UTR of the *cspA* mRNA than that with the wild type cells. The growth of cells harboring pUC19-600 or pUC19-2G (see Fig. 3) was indeed severely inhibited. This was characterized

by a longer lag period (data not shown).

EXAMPLE 6: Effects of the overproduction of *cspA*

The hyper-expression of the 5' UTR of the *cspA* mRNA resulted in the prolonged overproduction of CspA (see Fig. 2). Therefore, the effects observed above may be due to the overproduction of the CspA protein rather than the 5' UTR of the *cspA* mRNA. This possibility was examined using CL83 cells harboring pJJG02 which contains the entire *cspA* gene. Pulse-labeling experiments were carried out as described above. As shown in Fig. 7, with strain CL83 carrying pUC19, the expression of *cspA* and *csdA* (the gene for protein X) were induced at 1 hr after the temperature shift to 15°C (lanes 1 and 2), and returned to a basal level at 3 hr after the temperature shift (lane 3). On the other hand, when the cells were transformed with pJJG02, the expression of *cspA* was not only induced at 15°C, but also significantly higher than that of cells with pUC19 as judged by two-dimensional gel electrophoresis (not shown). It should be noted that high CspA production is still observed even at 3 hr 15°C (lane 6). Although this overproduction of CspA at 3 hr after cold-shock was very similar to the case with the overproduction of the 5' UTR of *cspA* as described earlier (Fig. 2), it is important to note that no prolonged lag period of cell growth and no prolonged production of other cold-shock proteins such as CspB and CsdA were observed at the same time point. These results indicate that the co-production of CspA with the 5' UTR of the *cspA* mRNA suppresses the effects of the overproduction of only the 5' UTR, and that the high levels of CspA production even at 3 hr after cold-shock are not the cause of this effect.

EXAMPLE 7: Identification of a repressor binding site

We attempted to identify the specific region responsible for the *cspA* derepression within the 5' UTR of *cspA* mRNA. Because of the 11-base sequence commonly found in the 5' UTR in *cspA*, *cspB* and *csdA*, the region containing this sequence from position +1 to +25 of the 5'UTR of the *cspA* mRNA was tested. This region was put under the control of the *cspA* promoter in pUC19 to construct p2JTEK (Fig. 8). Cells harboring p2JTEK were then examined for the pattern of total protein synthesis at 3 hr after the temperature shift from 37 to 15°C. As shown in lane 3 in Fig. 8, CspA and CsdA production was clearly derepressed, with concomitant inhibition of other cellular proteins, as evident from the lighter background in comparison with cells harboring pUC19 (Fig. 8, lane 1). Cells harboring p6mTEK, which transcribes only the region from +1 to +6 of the *cspA* mRNA showed a pattern (Fig. 8, lane 2) identical to that of cells with pUC19 (Fig. 8, lane 1). This result indicates that the region responsible for the *cspA* derepression resides within the first 25-base sequence of the 5' UTR of *cspA* mRNA.

EXAMPLE 8: Deletion Analysis of the *cspA* 5'-UTR

Previously, we constructed two *cspA-lacZ* fusions in which the *lacZ* gene was transcriptionally fused to *cspA* at +26 (pKM67; Mitta *et al.*, 1997) or at +143 (pJJG78; Jiang *et al.*, 1996) of the *cspA* mRNA. The β -galactosidase activity of the cells harboring pJJG78 was very low at 37°C, and increased about 10 fold at 2 h after temperature downshift to 15°C, whereas the β -galactosidase activity of the cells harboring pKM67 was very high even at 37°C (Mitta *et al.*, 1997). It has been proposed that the region from +26 to +143 in the 5'-UTR of *cspA* mRNA has three possible functions on cold-shock induction: (I) repression of *cspA* expression at 37°C, (ii) stabilization of its mRNA upon cold shock, and (iii) translation efficiency at 15°C (Mitta *et al.*, 1997).

In order to further investigate which part of the 5'-UTR is responsible for the positive or negative regulation of *cspA* expression, we attempted deletion analysis of the 5'-UTR and examined the effect of various deletions on cold-shock induction of *cspA*. For this purpose, a series of 5'-UTR deletion mutants were constructed, in which a 26- to 32-base deletion was created at every about 30 bases, and the resultant 5'-UTR was translationally fused to *lacZ* at the 13th amino acid residue of CspA. The resultant plasmids, pMM022, pMM023, pMM024, pMM025 and pMM026 contain deletion mutations in the 5'-UTR from +2 to +27, from +28 to +55, from +56 to +86, from +86 to +117, and from +118 to +143, respectively (Fig. 9A). In the case of pMM024, a deletion from +56 to +85 was originally designed, but all the transformants we analyzed contained an extra base deletion at position +86. Plasmid pMM67 (Mitta *et al.*, 1997), which is the wild-type *cspA-lacZ* translational fusion construct, was used as a control. *E. coli* AR137, a *pcnB* mutant, which is known to maintain pBR322 derivatives in a low copy number (Lopilato *et al.*, 1986), was used as a host for transformation in order to avoid multicopy effects of the constructed gene on their expression.

Transformed cells were grown in M9-Casamino acids medium at 37°C, and β -galactosidase activities were measured after temperature downshift from 37°C to 15°C. At 37°C (zero time point in Fig. 9B), β -galactosidase activities were 10 fold higher in cells harboring pMM024 (Δ 56-86) and pMM025 (Δ 86-117) than in cells harboring the wild-type pMM67, while other deletion mutants [pMM022 (Δ 2-27), pMM023 (Δ 28-55) and pMM026 (Δ 118-143)] showed very low β -galactosidase activities. These results suggest that the 5'-UTR region from base +56 to +117 is involved in the repression of *cspA* expression at 37°C. Interestingly β -galactosidase activity increased almost 5 fold with pMM024 (Δ 56-86) after temperature downshift, while it increased only less than 2 fold with pMM025 (Δ 86-117), suggesting that the region deleted in pMM025 (Δ 86-117) plays an important role in cold-

shock induction of *cspA*. Similar to pMM025 (Δ 86-117), β -galactosidase activity with pMM023 (Δ 28-55) was poorly induced at low temperature. In particular, the region deleted in pMM026 (Δ 118-143) appears to play a crucial role in *cspA* expression at both high and low temperatures, since β -galactosidase activity was very low at both 37°C and 15°C (Fig. 9B). The deletion of the region from base +2 to +27 (pMM022) containing the cold-box sequence involved in *cspA* autoregulation (Jiang *et al.*, 1996) has little effect on the cold-shock induction of *cspA* as predicted (Fig. 9B).

EXAMPLE 9: Analyses of *cspA-lacZ* mRNA

As described above, all deletion mutations of the 5'-UTR except for pMM022 (Δ 2-27) affected on cold-shock induction of *cspA*. The *cspA* promoter is known to be active even at 37°C (Goldenberg *et al.*, 1997; Fang *et al.*, 1997; Mitta *et al.*, 1997). Since all the deletion constructs have the intact *cspA* promoter (Fig. 9A), transcription efficiencies of these constructs are likely to be identical. On the other hand, the *cspA* mRNA stability is significantly different depending on growth temperatures (Brandi *et al.*, 1996; Goldenberg *et al.*, 1996; Bae *et al.*, 1997; Fang *et al.*, 1997; Goldenberg *et al.*, 1997; Mitta *et al.*, 1997). Therefore the effect of the deletion mutations on the *cspA* expression at low temperature may be due to different mRNA stabilities of the constructs. To examine this aspect, the primer extension analysis was carried out to quantitate the amounts of the *cspA-lacZ* transcripts for each mutant at different time points after the addition of rifampicin at both 37°C and 15°C (Fig. 10A). Again, in order to avoid multicopy effects either positively or negatively, strain AR137, a *pcnB* mutant, was used. The amounts of transcripts at each time point were estimated by a phosphorimager, and the amounts of mRNA remained (per cent of the amount at zero time point) were plotted as shown in Fig. 10B. All the transcripts were unstable at

37°C with their half-lives estimated between 30 and 45 s. At 15°C, however, they became very stable with half-lives between 20 and 40 min. These half-lives are similar to those for the wild-type construct pMM67 obtained previously (Mitta *et al.*, 1997) as well as to those for the wild-type chromosomal *cspA* (Fang *et al.*, 1997; Goldenberg *et al.*, 1997). It is important to note that in contrast to the similar mRNA half-lives at low temperature, β -galactosidase activities induced at 15°C were widely varied among all these constructs as shown in Fig. 9B.

These results indicate that *cspA* induction efficiencies as measured by β -galactosidase activity are not correlated to the mRNA stability of each deletion construct, but rather to the amount of mRNA and/or its translation efficiency for each construct. Therefore, we next examined the amounts of mRNA for each construct at different time points after cold shock by the primer extension method. The results are shown in Fig. 11A and the amounts of transcripts were estimated by a phosphorimager. Their relative amounts were calculated using the amount of the transcript of pMM67 at zero time as 1 (Fig. 11B). At 37°C, the amounts of transcripts for pMM022 (Δ 2-27) and pMM024 (Δ 56-86) were very similar to that of the wild-type construct pMM67 (column 1 in Fig. 11B). It should be noted that β -galactosidase activity of pMM024 (Δ 56-86) at 37°C was more than 10 times higher than that of pMM022 (Δ 2-27) (see Fig. 9B). In the case of pMM023 (Δ 28-55), pMM025 (Δ 86-117) and pMM026 (Δ 118-143), the amounts of transcripts at 37°C are approximately half of that of pMM67. Again it should be noted that β -galactosidase activity of pMM025 (Δ 86-117) was 10 times higher than those of pMM023 (Δ 28-55) and pMM026 (Δ 118-143) (Fig. 9B). These results indicate that there is no correlation between the amounts of transcripts and the β -galactosidase activities at 37°C.

EXAMPLE 10: Translational Regulation by the 5'-UTR

After temperature downshift, the amounts of the *cspA-lacZ* mRNAs dramatically increased in all the constructs and the induction patterns are shown in Fig. 11A and 11B.

They showed very similar pattern in accumulation of the transcripts as that of the wild-type pMM67, such that the maximal induction was observed at 1 h after temperature downshift.

5 The patterns of mRNA levels were very similar between pMM67 and pMM024 ($\Delta 56-86$), while the others also showed a similar induction pattern although the amounts of their mRNAs were approximately a half of that of the pMM67 mRNA at each time point. Since the promoter activity of all the deletion constructs are considered to be the same, and in addition their mRNA stabilities were also very similar to that of the wild-type construct (Fig. 10B), lower amounts of mRNAs for all the deletion constructs except for pMM024 ($\Delta 56-86$) are probably due to their slower transcription elongation rate and/or transcription attenuation within the 5'-UTR. A remarkable finding was that the amounts of mRNA for pMM026 ($\Delta 118-143$) accumulated after cold shock were also identical to those for pMM022 ($\Delta 2-27$), pMM023 ($\Delta 28-55$) and pMM025 ($\Delta 86-117$) (see Fig. 11B). Nevertheless cold-shock 15 induction of β -galactosidase activity was extremely low for pMM026 ($\Delta 118-143$) throughout cold-shock treatment (Fig. 9B), indicating that the mRNA for this construct was very poorly translated. Relative translation efficiencies at 15°C was calculated for all the constructs from the increments of β -galactosidase activity during the cold shock and the amounts of mRNA (see the Brief Description of the Figures Legend to Fig. 11). As shown in Fig. 11C, the 20 translation efficiency of pMM026 ($\Delta 118-143$) mRNA was extremely poor and calculated to be 9.5% of that of pMM67 mRNA (Fig. 11C column 6). Thus, it is clear that the deletion from base +118 to +143 of the 5'-UTR negatively affected on translation. Besides pMM026 ($\Delta 118-143$), the translation efficiencies of the mRNAs of pMM023 ($\Delta 28-55$) and pMM025 ($\Delta 86-117$) were relatively low and calculated to be 54 and 36% of that of pMM67 mRNA,

respectively. These results clearly indicate that the translation efficiency of mRNA plays a crucial role in the regulation of *cspA* expression and that in particular, the region from base +118 to +143 of the 5'-UTR plays a major role in translation efficiency.

Nucleotide sequence comparison of the 5'-UTR regions of the cold-shock inducible genes, *cspA*, *cspB* and *cspG*, reveals that there is a 13-base sequence (from base +123 to +135 of *cspA*) conserved very well among these genes as shown in Fig. 12. Interestingly, these 13-base sequences are located immediately upstream of the Shine-Dalgarno sequence and contains a palindromic sequence to form a stable secondary structure ($\Delta G = -9.5\text{kcal}$; see Figs. 12 and 14). It is also complementary to the region from base 1023 to 1035 of 16S rRNA (Fig. 12). In the pMM026 ($\Delta 118-143$) mRNA, this upstream-box region is deleted, which may be the major cause for the poor translation efficiency of the mRNA.

In order to characterize the role of the upstream box in translation efficiency, two new constructs were made; in one construct (pKNJ37) the exact 13-base upstream-box sequence was deleted from the wild-type *cspA-lacZ* construct (pMM007) and in another (pKNJ38) the 13-base sequence was added at the upstream region of SD of pKM67 (Mitta *et al.*, 1997) as shown in Fig. 13A. In pKM67 the *lacZ* gene is fused at base +26, and it has been shown that as a result of the substantial deletion in the 5'UTR β -galactosidase became expressed even at 37°C without any further induction upon cold shock (Mitta *et al.*, 1997). Note that both pMM007 and pMM67 have the exactly identical insert of *cspA* in different vectors, pRS414 and pKM005, respectively. Cold-shock induction patterns of β -galactosidase activities of these two plasmids (pMM007 and pMM67) are similar (data not shown). Cells were transformed with pKNJ37, pMM007, pKNJ38 and pKM67, and cold-shock induction of β -galactosidase was examined. Deletion of the upstream box (pKNJ37) significantly lowered β -galactosidase activity not only at 37°C but also upon cold shock (Fig.13B). Contrary to the

result, when the upstream box was inserted into pKM67 13 bases upstream of the SD sequence, the constitutive expression of β -galactosidase at 37°C increased by approximately 20% (Fig.13B). This increment was also kept upon cold shock. These results support the notion that the upstream-box sequence is associated with efficient translation of *cspA*.

5 Specific sequences of the 5' UTR which are useful in the invention include 5'-GCCGAAAGGCACA-3' (SEQ ID NO:48), 5'-GCCGAAAGGCUCA-3' (SEQ ID NO:49), and 5'-GCCGAAAGGCCCA-3' (SEQ ID NO:50). (See Fig. 12).

It is currently considered that *cspA* expression is regulated at the levels of transcription, mRNA stability and translation as follows: (I) The *cspA* gene has a strong
10 promoter equipped with the UP element, which works at both 37°C and 15°C (Fang *et al.*, 1997; Goldenberg *et al.*, 1997; Mitta *et al.*, 1997), although transcription of the *cspA* gene does not require any *de novo* protein synthesis upon cold shock. (ii) The *cspA* mRNA contains the downstream-box (DB) sequence downstream of the initiation codon, which plays a major role in enhancement of translation initiation at low temperatures (Etchegaray and
15 Inouye, unpublished; Mitta *et al.*, 1997). (iii) It is very important to note that the *cspA* mRNA has an unusually long 5'-UTR (Tanabe *et al.*, 1992), consisting of 159 bases, and is extremely unstable at 37°C (Brandi *et al.*, 1996; Goldenberg *et al.*, 1996; Bae *et al.*, 1997; Fang *et al.*, 1997; Jiang *et al.*, 1997; Goldenberg *et al.*, 1997; Mitta *et al.*, 1997). Immediately upon cold shock, the *cspA* mRNA becomes stable. Again, this stabilization of mRNA upon cold
20 shock does not require any *de novo* protein synthesis. When the region from base +26 to +143 of the 5'-UTR was deleted from the *cspA-lacZ* fusion construct, high β -galactosidase activity was obtained even at 37°C (Mitta *et al.*, 1997). In the 5'-UTR, there is a putative RNaseE cleavage site immediately upstream of the SD sequence (Fang *et al.*, 1997). This site is considered to be responsible for the extreme instability of the *cspA* mRNA, since the three-

base substitution mutation at this region resulted in 150-fold stabilization of the mRNA, allowing a high CspA production even at 37°C (Fang *et al.*, 1997). Thus, it is clear that the unusually long 5'-UTR of the *cspA* mRNA is responsible for its instability at 37°C. It can be considered that the 5'-UTR makes the *cspA* mRNA extremely unstable at 37°C, in such a way as *cspA* is cold-shock inducible. It is worth mentioning that although the *cspA* mRNA becomes stable and is accumulated at the nonpermissive temperature in the temperature-sensitive RNaseE mutant, CspA production was not detected under this condition (Fang *et al.*, 1997), suggesting that in addition to the stability of mRNA another role may exist in the 5'-UTR.

We have attempted to further elucidate the roles of the unusually long 5'-UTR of the *cspA* mRNA in *cspA* expression. We made a series of 26- to 32-base deletion mutations encompassing the entire 5'-UTR. These mutated 5'-UTR regions were translationally fused to *lacZ* at the 13th amino acid residue of CspA after the DB sequence. At 37°C, pMM022 ($\Delta 2$ -27), pMM023 ($\Delta 28$ -55) and pMM026 ($\Delta 118$ -143) showed the similar β -galactosidase activities as the wild-type pMM67, while pMM024 ($\Delta 56$ -86) and pMM025 ($\Delta 86$ -117) showed more than 10 fold higher β -galactosidase activities than pMM67, indicating that the region from base +56 to +117 is involved in the repression of *cspA* expression at 37°C.

Based on the β -galactosidase activities after temperature downshift, mutants can be classified into three classes; those in Class I [pMM022 ($\Delta 2$ -27) and pMM024 ($\Delta 56$ -86)] in which β -galactosidase is induced in a similar fashion to pMM67, those in Class II [pMM023 ($\Delta 28$ -55) and pMM025 ($\Delta 86$ -117)] in which cold-shock induction of β -galactosidase is poor, and that in Class III [pMM026 ($\Delta 118$ -143)] with very low β -galactosidase activities both before and after cold shock. It is worth mentioning that these differences were due to neither the amounts nor the stability of mRNA as evident from Figs. 10 and 11. This supports the

notion that the 5'-UTR has another role in translation efficiency in addition to the stability of mRNA as mentioned above.

The relative translation efficiencies after temperature downshift for different constructs showed surprisingly significant differences (Fig. 11C), which coincide well with the classification of the constructs at 15°C as described above. The translation efficiencies with pMM022 ($\Delta 2-27$) and pMM024 ($\Delta 56-86$) (Class I) are a little better than that of the wild-type pMM67, those with pMM023 ($\Delta 28-55$) and pMM025 ($\Delta 86-117$) (Class II) are 40 to 50% of that of pMM67, and that with pMM026 ($\Delta 118-143$) (Class III) is less than 10% of that of pMM67.

In pMM026 ($\Delta 118-143$), the deletion mutation is clearly affecting the translation efficiency but not the stability of mRNA. The deleted region was found to contain a 13-base sequence (base +123 to +135) well conserved in the mRNAs for all the cold-shock inducible *cspA* family, *cspA*, *cspB*, *cspG*, and *cspI* (see Fig. 12). This sequence designated the upstream box may form a distinct secondary structure in both the wild-type pMM67 and Class I constructs [pMM022 ($\Delta 2-27$) and pMM024 ($\Delta 56-86$)] (Fig. 14). Class I constructs showed a similar translation efficiency to the wild-type. In Class II [pMM023 ($\Delta 28-55$) and pMM025 ($\Delta 86-117$)], which showed 50% of translation efficiency of the wild-type, this secondary structure disappears, however, the predicted secondary structures around the SD sequence in these constructs are still similar to that of the wild-type construct. In contrast, when the upstream-box region is deleted [pMM026 ($\Delta 118-143$); Class III, which showed a very poor translation efficiency], the SD region forms a more stable secondary structure. This likely prevents recognition of the mRNA by ribosomes, causing a very poor translation efficiency in pMM026 ($\Delta 118-143$). Therefore the upstream box may function to punctuate the formation of a stable secondary structure immediately upstream of the SD sequence,

allowing it highly accessible to ribosomes. Alternatively, as the upstream-box sequence is complementary to the 16S rRNA sequence from base 1023 to 1035 (see Fig. 12), it is possible that the upstream-box sequence may be another *cis*-element, which may enhance translation efficiency by forming a duplex with 16S rRNA in addition to the SD sequence and the downstream box (Mitta *et al.* 1997). Since all the constructs use the identical site of *cspA* to fuse to *lacZ*, the observed differences in translation efficiency are considered to be at the level of translation initiation but not at the level of translation elongation. It has been suggested that such an interaction between mRNA and 16S rRNA plays an important role in translation initiation (McCarthy and Brimacombe, 1994). The region from base 1023 to 1035 of 16S rRNA has been shown to be near the site where 30S ribosomes interact with mRNA on the basis of the fact that the U residue at position 1052 directly interacts with the 6th base from the initiation codon (Dontsova *et al.*, 1992; Rinke-Appel *et al.*, 1993). Furthermore, it has been reported that RNase VI sensitivity at G1020, A1021 and A1022 increased immediately after the assembly of 30S ribosomal subunits, indicating that the site encompassing these residues is exposed to the surface (Powers *et al.*, 1988).

Consistent with the proposed role of the upstream box in the translation, the addition of an upstream-box sequence to pKM67 resulted in increase of β -galactosidase activity approximately by 20%. On the other hand, the deletion of the exact 13-base upstream-box sequence [pKNJ37 (Δ 123-135) in Fig. 13] resulted in 50% reduction of β -galactosidase activity at 37°C and a lower level of induction upon cold shock. Note that the predicted secondary structure surrounding the SD sequence of pKNJ37 (Δ 123-135) is the same as the wild-type (Fig. 14).

In the heat shock response, the synthesis of the heat-shock sigma factor, σ^{32} , is regulated at the level of translation and the secondary structure of the *rpoH* mRNA plays a

crucial role in this regulation (Nagi *et al.*, 1991; Yuzawa *et al.*, 1993). Very recently, the secondary structure of the *rpoH* mRNA has been determined by chemical and enzymatic probing assays and the results are completely consistent with the predicted *rpoH* secondary structure proposed previously (Morita *et al.*, 1999). It has also been shown that mutations in the *rpoH* mRNA, which are predicted to decrease the mRNA stability, increased *rpoH* expression and *vice versa* (Morita *et al.*, 1999). Toeprinting assays using the wild-type *rpoH* mRNA and 30S ribosomes showed that the toeprinting appearance totally depends on temperature, indicating that the *rpoH* mRNA secondary structure at 42°C, which is accessible to ribosomes, is different from that at 30°C, and that the *rpoH* mRNA structure itself is a determinant for *rpoH* expression without any other factors (Morita *et al.*, 1999). Thus, it was proposed that the *rpoH* mRNA acts as an RNA thermosensor (Morita *et al.*, 1999). Storz (1999) discussed that the *lcrF* mRNA of *Yersinia pestis* (Hoe *et al.*, 1993) and the λ phage cIII mRNA (Altuvia *et al.*, 1989) might be other examples for RNA thermosensor.

In summary, stabilization of the *cspA* mRNA upon cold shock is prerequisite for CspA production. This stabilization does not require any *de novo* protein synthesis. As presented here, translation efficiency, which may involve the secondary structure of the 5'-UTR, in particular surrounding the SD sequence, of *cspA* mRNA, is turned out to play an important role in *cspA* expression in addition to the mRNA stabilization. It is thus possible that the *cspA* mRNA might act as an RNA thermometer as proposed for the *rpoH* mRNA. To know the more precise molecular mechanism of the regulation of *cspA* expression, determination of the secondary structure of the *cspA* mRNA and the relationship between the secondary structure and the translation efficiency remain to be addressed. The point mutation analysis in addition to the deletion analysis presented here will give us information on the molecular anatomy of the structure and function of 5'-UTR of the *cspA* mRNA.

Example 11: The Role of DB in the Cold-shock Induction of *cspB*, and the Effect of a Perfectly Matching DB

Previously, we have demonstrated that the expression of *cspB* at low temperature is primarily regulated at the mRNA level (Etchegaray et al., 1996). Here, we first show that DB
5 is an important contributor to the translational induction of *cspB* at low temperature. *E. coli* AR137, a *pcnB* mutant which maintains pBR322 derivative plasmids at low copy (Lopilato et al., 1986) was transformed with a series of *cspB-lacZ* translational fusions (Fig. 15A and 15B). Figure 15C shows β -galactosidase activity at various time points after temperature shift from 37 to 15°C. After 3 hr at 15°C, pB13 and pB17 show the highest levels of β -
10 galactosidase activity, indicating that a region from codon 3 and 13 (containing DB) plays a major role in high expression of *cspB* at low temperature. Levels of β -galactosidase units from the SD-deletion constructs pB13sd and pB17sd demonstrate that SD is also required for the induction of *cspB*. Primer extension analysis (Fig. 15D) shows that the mRNA levels of pB3 and pB17 are almost identical, while the β -galactosidase activity of pB17 is 7 times
15 higher than that of pB3 (Fig. 15C), demonstrating that the differences in the sequence downstream of the initiation codon cause a significant effect on the efficacy of mRNA translation but not on the amount of the mRNAs. Based on the amounts of mRNA estimated using a phosphorimager and the increments of β -galactosidase activity between 1 and 2 hr. after cold-shock induction, the translational capability of pB17 was calculated to be 6 times
20 higher than that of pB13 and 18 times higher than that of pB3. This could be explained by the potential to form additional base-pairing between the *cspB*-mRNA (pB17) and the 16S rRNA as shown in Figure 15A. This suggests that a longer DB (pB17) could be more effective for translation than a shorter DB (pB13). When the SD sequence was deleted from

pB13, the translational efficiency became even lower than that of pB3, indicating that both SD and DB are required for the full expression of *cspB* at low temperature.

It has been shown that mRNA stability plays a role in the cold-shock inducibility of *cspA* (Brandi *et al.*, 1996; Goldenberg *et al.*, 1997; Fang *et al.*, 1997). However, the dramatic effect of DB on the *lacZ* expression cannot account for the differences of the mRNA stabilities at 15°C between the constructs with and without DB. Fig. 15E shows the half-life for pB3, pB13, pB13sd and pB17 to be 12, 22, 15 and 20 minutes, respectively.

It has been shown that mRNA stability plays a role in the cold-shock inducibility of *cspA* (Brandi *et al.*, 1996; Goldenberg *et al.*, 1997; Fang *et al.*, 1997). However, the dramatic effect of DB on the *lacZ* expression cannot account for the differences of the mRNA stabilities at 15°C between the constructs with and without DB. Figure 1E shows the half life for pB3, pB13, pB13sd, and pB17 to be 12, 22, 15 and 20 minutes, respectively.

It has been previously shown that DB is essential for the production of CspA at low temperature (Mitta *et al.*, 1997). However, the wild-type DB of *cspA* has 10 matches out of 15 possible matches to the anti-DB in 16S rRNA (Mitta *et al.*, 1997). Therefore, we added DBs of 12 (pJJG78DB1) or 15 (pJJG78DB2) bases that are complementary with the anti-DB of 16S rRNA to the site after the 5th codon of *lacZ* under the *cspA* regulatory system in pJJG78 (see Fig. 15A) to examine if they enhance *lacZ* expression at 15°C. Mid-log phase cells (*pcnB*⁻) grown at 37°C were shifted to 15°C and β-galactosidase activity was measured at 1, 2 and 3 hr after the shift. Fig. 15B shows that at 1 hr at 15°C the β-galactosidase activity was 3 and 8 fold higher with pJJG78DB1 and pJJG78DB2, respectively than with pJJG78. After 2 and 3 hr at 15°C, the β-galactosidase activity was increased 3.5 and 10.5 times with pJJG78DB1 and pJJG78DB2, respectively than with pJJG78. Moreover, the effect of the DB was observed at 37°C in which the β-galactosidase activity of pJJG78DB1 and pJJG78DB2

was 2 and 4 fold higher as compared with pJIG78. The amount of the *lacZ* mRNA (Fig. 15C) as well as the mRNA stability did not vary significantly between these constructs. The *lacZ* mRNA half-life from pJIG78, pJIG78DB1 and pJIG78DB2 was calculated to be 27, 23 and 25 min respectively. In addition, computer analysis (Zuker and Stieger 1982) revealed no significant differences in the mRNA secondary structures among pJIG78, pJIG78DB1 and pJIG78DB2, suggesting that the insertion of the perfectly matching DB may not have a particular effect in the mRNA secondary structures that could account for the difference in their β -galactosidase expression. These results indicate that DB function as a translational enhancer and that greater complementarity to the anti-DB improves translational efficiency and/or that specific base-pairings like the first 3 nucleotides of the DB from pJIG78DB2 may play an important role for the DB activity.

Example 12: Analysis of DB Function

The experiments described above were carried out at 15°C. In order to examine whether DB also works at 37°C, the *cspA* cold-shock regulatory regions upstream of SD of pJIG78 and pJIG78DB2 were replaced with the constitutive *lpp* promoter and the *lac* promoter-operator fragment using a pINIII vector (Inouye, M. 1983), yielding pINZ and pINZDB1, respectively (Fig. 17A). Cells (*pcnB*⁻) transformed with pINZ or pINZDB1 showed very low β -galactosidase activity in the absence of IPTG, an inducer of the *lac* promoter (Fig. 1C, time 0). Upon the addition of 1mM IPTG, β -galactosidase activity was induced in both cells. After 3 hr induction, β -galactosidase activity increased 18 and 37 folds for pINZ and pINZDB1, respectively (Fig. 17C). However, the levels of β -galactosidase activity show a dramatic difference between the two; the activity with DB (pINZDB1) was 34 times higher than that without DB (pINZ), demonstrating that DB functions at 37°C as well.

Specific activities of β -galactosidase produced from vector pINZ and pINZDB1 are almost identical (data not shown) and thus the addition of the 5 amino acid residues in the β -galactosidase sequence of pINZDB1 (due to DB) does not affect the enzymatic activity. Furthermore, the stabilities of β -galactosidase from pINZ and pINZDB1 are also identical with a half-life of approximately 3 hr (data not shown).

Next, we examined whether DB functions independently from SD, the initial ribosomebinding site. For this purpose, the SD sequence of pINZDB1, GAGG was changed to GCCC, yielding pINZDB2 (Figure 17A and 17B). The β -galactosidase level of pINZDB2 induction was reduced to 1/7 of that of pINZDB1, but still 3 fold higher than that of pINZ at 3 hr after IPTG induction (Figure 17C). However, since pINZDB2 has the second AUG codon 6 codons downstream as a result of DB insertion it might serve as a secondary initiation codon for the *lacZ* gene. Indeed, the N-terminal sequence analysis showed that 100% of the β -galactosidase produced from pINZDB2 is initiated at the second AUG codon as compared with pINZDB1, which is more than 90% initiated at the first AUG codon (data not shown). Furthermore, the second AUG codon is preceded by a potential but poor SD sequence (AAGG) at the region corresponding to the 2nd and 3th codons (underlined; Fig. 17B). Indeed, when this secondary SD was removed by deletion of the 15-base sequence (codon 1 to 5; pINZDB3 in Fig. 17B), β -galactosidase activity at all time points was reduced to the background level (Fig. 17C), indicating that the secondary SD played a crucial role in the translation of the pINZDB2 *lacZ* mRNA. When the SD sequence was recreated by 5 base substitution in pINZDB3 (pINZDB4; Fig. 17B), β -galactosidase activity of this construct was recovered to a comparable level to that of pINZDB1 (Fig. 17C). It is important to notice that the DB sequence starting from the first AUG codon was eliminated in pINZDB4 (Fig. 17B). Therefore, the high expression of β -galactosidase from pINZDB1 and pINZDB4 is due to the

perfectly matching DB sequence (Fig. 17B). These results indicate that (a) DB functions only in the presence of SD, (b) the position of DB is flexible starting from either codon 1 or 6.

Example 13: Enhancement of Translation by DB

5 The β -galactosidase activity showed in Fig. 17C indicates that DB enhances the translation of pINZDB1. Therefore, in order to test the effect the DB in translation efficiency the rate of β -galactosidase synthesis from pINZ and pINZDB1 was analyzed. The rate of β -galactosidase synthesis was measured by pulse-labeling cells for 5 min with [35 S]-methionine after the addition of IPTG using cells harboring pINZ and pINZDB1. After SDS-PAGE, the
10 amounts of radioactive β -galactosidase were estimated using a phosphorimager (Fig. 17D). Prior to the addition of IPTG, the rate of β -galactosidase synthesis from pINZ and pINZDB1 was identical. However, upon IPTG induction the rates of β -galactosidase synthesis from pINZDB1 was continuously increasing at each time point while the rate of β -galactosidase synthesis from pINZ was almost non affected. After 4 hr of IPTG addition the rate of β -
15 galactosidase synthesis from pINZDB1 was 6.5 times higher than that of pINZ. This result demonstrate that DB enhances the translation efficiency of pINZDB1 as reflected by the increment in the synthesis of β -galactosidase.

 In order to examine whether DB enhances translation initiation we next analyze the ability of *lacZ* mRNA from pINZ and pINZDB1 to form polysomes. For this experiment,
20 *pcnB*⁺ cells were used to amplify the effect of DB. Interestingly, cells with pINZDB1 could not form colonies on LB plates in the presence of 1 mM IPTG, while cells with pINZ formed colonies. The lethal effect of IPTG on the cells with pINZDB1 is considered to be due to overexpression of β -galactosidase. After the addition of IPTG, cell growth was stopped by the addition of chloramphenicol (0.1 mg/ml) at 15, 30 and 60 min and then polysome profiles

were examined as shown in Fig. 17D. From each gradient fraction (500 ml), 200 ml were spotted on a nitrocellulose membrane and the amount of the *lacZ* mRNA analyzed using a 24-base antisense oligonucleotide (M13-47 oligonucleotide). The amounts of the *lacZ* mRNA were quantified by a phosphorimager and are displayed in Fig. 17D. While the polysome profiles are similar, there are significant differences in the distribution of the *lacZ* mRNA; at 15 min the *lacZ* mRNA mainly exists in the upper half of the gradient (fraction 8 to 14, corresponding to 70S to 30S ribosomes) with pINZ, while with pINZDB1 a major peak (fraction 3 to 8) is formed in the lower half of the gradient. At 30 min, the *lacZ* mRNA from pINZ moved to the position of 70S ribosome, while the *lacZ* mRNA from pINZDB1 maintained a similar pattern as that at 15 min. At 60 min a major fraction of the *lacZ* mRNA from pINZ remained in the upper half of the gradient, while the *lacZ* mRNA from pINZDB1 was broadly distributed from higher order polysomes to 70S ribosome fraction. Therefore, the reason why cells harboring pINZDB1 could not form colonies on LB plates containing 1 mM IPTG may be due to a decrease in the concentration of free ribosomes as a result of the massive expression of a highly translatable DB-containing mRNA (Vind *et al.*, 1993). These results indicate that DB enhances the efficiency of polysome formation probably due to a translation initiation enhancement.

In order to estimate the exact effect of DB from the above experiment, the amount of the *lacZ* mRNA and the β -galactosidase activity were measured at the same time points taken in the polysome profiles (15, 30 and 60 min after IPTG induction). As shown in Fig. 18A, the amounts of the *lacZ* mRNA reached almost the maximal level at 15 min for both pINZ and pINZDB1. The phosphorimager analysis of this result revealed that the amounts of the pINZDB1 mRNA are 1.5, 1.4 and 1.3 times higher than those of the pINZ mRNA at 15, 30 and 60 min, respectively. The higher mRNA levels for pINZDB1 are probably attributable to

the highly efficient polysome formation of pINZDB1 that may stabilize the mRNA (Iost and Dreyfus 1995). The induction of β -galactosidase activity is shown in Fig. 18B. In the case of pINZDB1, the activity is very high even in the absence of IPTG, and upon the addition of IPTG, it increased from 18,500 to 64,400 units (3.5 fold) after 2.5 hr incubation. In the case of pINZ, the background activity prior to IPTG induction was much lower, and it increased from 900 to 2,900 units (3 fold) at the 2.5 hr time point. The increment of the β -galactosidase activity of pINZDB1 between 30 and 60 min is 35 times higher than that of pINZ, and therefore the efficiency of β -galactosidase production for pINZDB1 is calculated to be 26 times higher than that for pINZ on the bases of the amount of mRNA. Therefore, the higher levels of β -galactosidase production from pINZDB1 are due to a high efficiency of polysome formation.

Next, in order to more directly demonstrate the translation-enhancement effect of DB the β -galactosidase synthesis was examined in a cell-free system using pINZ and pINZDB1. The [35 S]methionine incorporation into β -galactosidase (band G) with pINZDB1 (lane 2, Fig. 19) was 8 fold higher than that with pINZ (lane 1), while the β -lactamase (band L) production was almost identical in both lanes.

It has been proposed that in the absence of ribosomal protein S2, structural changes in 16S rRNA result in the release of the anti-DB sequence from the penultimate stem making it more accessible to base-pair with DB (Shean and Gottesman 1992; Powers *et al.*, 1988). We analyzed the β -galactosidase expression of pINZ, pINZDB1 in *E. coli* CS239 that carries an S2 temperature-sensitive mutation (Shean and Gottesman, 1992). Fig. 20A shows that the β -galactosidase activity of pINZDB1 significantly increases upon shifting the temperature from 30 to 42°C in the S2^{ts} strain (CS239) (6.3 fold from 0 to 3.5 hr), while the activity in the wild type strain (CS240) slightly increased (1.1 fold from 0 to 3.5 hr). If the initial ratio of the

activity of CS239 to that of CS240 at time 0 is taken as one, the ratio dramatically increased, reaching 5.8 at 3.5 hr after temperature shift (Fig. 20B). In contrast, the *lacZ* gene without DB did not show any significant differences in its expression between CS240 and CS239, and the ratios of the activity of CS239 to that of CS240 remained also at the initial level throughout the incubation time (Fig. 20B). A similar experiment was carried out with pINZDB3 (SD⁻, DB⁺), and the ratio of the activity in CS239 to that in CS240 increased 3.4 fold at 3.5 hr after the temperature shift (data not shown). These results clearly demonstrate that the low levels of S2 protein at 42°C causes significant stimulation of the *lacZ* expression only if the *lacZ* gene contains DB, consistent to the proposal of Shean and Gottesman (1992).

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IN THE EUROPEAN PATENT OFFICE
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For: COLD-SHOCK REGULATORY ELEMENTS, CONSTRUCTS THEREOF, AND
METHODS OF USE

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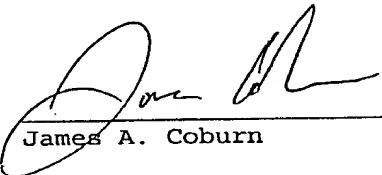
STATEMENT ACCOMPANYING SEQUENCE LISTING

Dear Sir:

The undersigned hereby states that the Sequence Listing submitted concurrently herewith does not include matter which goes beyond the content of the application as filed and that the information recorded on the diskette submitted concurrently herewith is identical to the written Sequence Listing.

Respectfully submitted,

Dec. 17, 1999
Date


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